Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 68 JUNE, 1948 No. 2

SECTION MEETINGS

CLEVELAND	
Western Reserve University	May 14, 1948
MINNESOTA	
University of Minnesota	May 12, 1948
NEW YORK	
New York Academy of Medicine	May 5, 1948
PACIFIC COAST	
Stanford University	April 17, 1948
WESTERN NEW YORK	
University of Buffalo	April 24, 1948
DISTRICT OF COLUMBIA	
George Washington University Medical School	April 1, 1948
ILLINOIS	
Michael Reese Hospital, Chicago	May 25, 1948
SOUTHERN	
Tulane University Medical School	May 14, 1948

16439

Activation of Prothrombin by Platelets Plus Globulin.*

J. H. MILSTONE.† (Introduced by M. C. Winternitz.)

From Laboratory of Pathology, Yale University School of Medicine, New Haven, Conn.

The clot-promoting effect of platelet suspensions is attributed to the platelets themselves, rather than to accompanying blood components. The mode of this platelet action still remains to be clarified. It is often stated that platelets plus calcium ions activate prothrombin. But it is obvious that the facts are not that simple; for platelets, calcium and prothrombin coexist in the circulating blood. It seems that at least one other factor must be involved. The following experiments differ from previous two-stage tests in that a globulin co-factor has been removed from both the platelets and the prothrombin, and then restored to the system for the prothrombin activation tests.

Materials and Methods. In general, the materials and methods were those which have been described,[1] except that the buffered saline contained 9 g NaCl, 200 ml 0.1 M sodium diethylbarbiturate and 144 ml 0.1 M HCl per liter of solution.

Twenty-five ml rabbit blood was drawn from the heart into a syringe containing 25

---

* Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

† This work was done during the tenure of a Life Insurance Medical Research Fellowship.

ml cold acid-citrate (37.5 g $2\ Na_3C_6H_5O_7.\ 11\ H_2O$ plus 1.05 g $H_3C_6H_5O_7 \cdot H_2O$ per liter of solution, pH 6.3). The platelets were separated by 3 cycles of differential centrifugation during which they were washed twice with cold buffered saline, pH 7.4. The centrifugations were carried out in 9½ x 1½ cm lusteroid tubes. The 3 slow centrifugations were at 1100 r.p.m. in an International, Size 1, Type C, centrifuge at room temperature; whereas the 3 alternate fast runs were made at 3400 r.p.m. in a Servall, Type SP angle centrifuge kept in a refrigerator at 2-4°C. After the first slow 20-minute run, the turbid plasma was pipetted off and the red cells were discarded. A 30-minute fast run served to sediment the platelets out of the plasma, along with some red and white cells. The sediment was suspended in 20 ml cold buffered saline with the aid of a glass pipette, and the suspension was centrifuged slowly for 10 minutes to remove most of the remaining red cells, along with some platelets. The turbid supernatant was subjected to a 20-minute fast centrifugation, and the resulting sediment was resuspended in 20 ml cold buffered saline. The ensuing fifth and sixth runs were like the third and fourth. The final platelet sediment was suspended in 2.5 ml buffered saline. Microscopic examination revealed one red cell per several hundred platelets. No white cells were found. Wright stains showed platelets with the characteristic reddish purple granules, but the blue hyalomere was observed only occasionally.

All biologic materials except the platelets were bovine. The crude globulin was prepared by adding calcium chloride to a plasma euglobulin solution, removing the resultant fibrin and allowing the solution to age in the refrigerator. Its thrombin was just detectable in the dilutions used. The factor or factors significant for the experiments that follow, although of unknown nature, will be referred to as "globulin."

All activation experiments were performed in glass, at room temperature, in the presence of calcium chloride. The molarity of the latter was usually in the neighborhood of 0.0025 M.

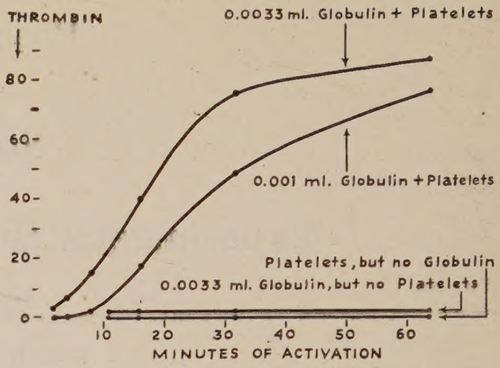


Fig. 1.

Activation of prothrombin by platelets, calcium, and globulin. 0.3 ml buffer, 0.4 ml prothrombin, 0.1 ml platelets, 0.1 ml Ca 0.0275 M, and 0.1 ml globulin dilution (1/30 or 1/100). Ca added 15 seconds before globulin. Activation timed from the addition of globulin. The height of the "Globulin but no Platelets" curve reflects the amount of thrombin in the globulin preparation.

Experimental. The platelets were studied microscopically as they aged in buffered saline without added anticoagulant. When suspensions were kept at room temperature in a sealed hanging drop preparation, the platelets lost their refractility slowly over a period of several hours; concomitantly they became swollen and less distinct in outline. These changes were only a little faster, if at all, when calcium chloride was added. Rapid disruption of twice-washed platelets in the presence of calcium and in contact with glass was not observed.

Although the behavior of different platelet suspensions varied, activation of prothrombin by fresh, twice-washed platelets was always delayed. Production of thrombin was much faster when a small amount of globulin was included. In the two-stage tests of Fig. 1, platelets plus globulin activated prothrombin whereas neither the platelet suspension nor the globulin was efficient alone. On prolonged incubation, some activation occurred with platelets, little, if any, with globulin. Platelets plus globulin did not yield thrombin unless prothrombin was added. The quantity of platelets used for 1 ml activation mixture was derived from 1 ml whole blood. The quantity of globulin used for the highest curve of Fig. 1 was derived from about 0.005 ml blood, and represented less than one-hundredth the amount of globulin used as thrombokinase in a previous study.[1] In these estimates, no allowance was made for large losses during preparation.

The effectiveness of the platelet-globulin system was much reduced by heating either component at 70°C for 10 minutes. A suspension of kaolin could not be substituted for the platelet suspension, although the same kaolin suspension was able to hasten the production of thrombin in a cruder test system.

When a platelet sediment was suspended in distilled water, it was at once less turbid than a similar sediment suspended in buffered saline. The water-clear extract obtained with distilled water behaved like the suspension of Fig. 1, although not all of the original activity was recovered in the extract. Merely setting the platelet factor free was not enough to enable it to activate prothrombin promptly. The presence of the globulin was still necessary.

Varying the amount of platelets and of globulin independently showed that the rate of thrombin formation was determined by both factors but was strictly proportional to neither. That the effects of the two components were not simply additive was further indicated by the fact that much faster activation was obtained with platelets and globulin than was obtained with eight times as much platelet suspension without globulin. When the quantity of platelets was raised above a certain level, activation was retarded.

Supplementary tests with dog platelets showed considerable quantitative differences, but the basic platelet-globulin relationship was maintained. Future work with all materials from the same species is contemplated.

Three-stage tests[1] were used to follow the thrombokinase activity of platelet-globulin mixtures. Although some results were suggestive, they were generally too erratic to permit the conclusion that thrombokinase activity was being developed progressively. Rapid inactivation of thrombokinase was sometimes apparent. This complication may have contributed to the capricious results obtained with the three-stage analyses, and also to the equivocal quantitative relationships observed in the two-stage tests.

Discussion. It has been shown that a suspension of washed platelets which induced very slow activation of prothrombin was not therefore of low potency. Such a suspension displayed considerable activity when tested in conjunction with a small quantity of a globulin preparation. The latter preparation, in turn, showed little if any thrombokinase activity, in the dilutions used, when tested without platelets.

Conceivably, platelets might contain prothrombokinase as suggested by Collingwood and MacMahon,[2] or a platelet enzyme might activate the prothrombokinase ("thromboplastinogen") of plasma, as suggested by Quick.[3] The present experiments do not decide between these hypotheses, nor do they eliminate other possibilities.

Lenggenhager[4,5] believed that the effect of platelets on blood coagulation was entirely due to their particulate nature, and consequent surface activity, and reported that kaolin particles had similar effects. The function of the platelets as defined by the present system was probably due to a substance derived from the platelets, and not to such surface effects, because the platelet suspensions could be replaced by platelet extracts but not by kaolin. Some possibility remains that the substance was adsorbed on the platelets or associated with inconspicuous accompanying particles, and although not completely removed by two washes, it was set free by the treatment with distilled water. Finally, the questions of particulate nature and surface activity recur at another level, when it is considered that the coagulant substance in the platelet extracts might have been in the form of particulates of macromolecular dimensions.

---

[1] Milstone, J. H., *J. Gen. Physiol.*, 1948, **31**, 301.

[2] Collingwood, B. J., and MacMahon, M. T., *J. Physiol.*, 1912, **45**, 119.

[3] Quick, A. J., *Am. J. Med. Sc.*, 1947, **214**, 272.

[4] Lenggenhager, K., *Klin. Woch.*, 1936, **15**, 1835.

[5] Lenggenhager, K., *Helv. Med. Acta*, 1940, **7**, 262.

The early work of Wright and Minot[6] showed that a factor associated with the serum globulins was important in bringing about the metamorphosis and disintegration of platelets in the presence of calcium. This would account for the relative stability of washed platelets. It will be of interest to inquire how the globulin factor as detected by platelet metamorphosis is related to the substance or substances which function as co-factor for platelets in the activation of prothrombin.

Summary. 1. Twice-washed platelets were relatively stable in the presence of calcium ions and in contact with glass.

2. In the presence of calcium, washed rabbit platelets plus a small amount of bovine globulin activated bovine prothrombin. Activation was much slower with platelets alone, and no activation was detected with the globulin alone.

3. Water-clear platelet extracts could be substituted for platelet suspensions in this test system. In such case, the presence of the globulin was still necessary for prompt activation of prothrombin.

---

[6] Wright, J. H., and Minot, G. R., *J. Exp. Med.*, 1917, **26**, 395.

16440

The Rickettsiostatic Action of Crystalline Penicillin Fractions in Embryonate Eggs.*

DONALD GREIFF AND HENRY PINKERTON.

From the Departments of Biology and Pathology, St. Louis University.

In 1944, we found that crude commercial penicillin strikingly inhibited the growth of typhus rickettsiae in the yolk sac,[1] and markedly reduced or even completely prevented the mortality from typhus infection in mice.[2] In fertile eggs, the total dosage used was 975 units per egg, given in 3 doses on the 2nd, 4th and 6th, or on the 3rd, 5th and 7th days after rickettsial injection. Doses of 100 units had little or no effect.[3] In mice, the most impressive results were obtained with daily doses ranging from 640 to 1100 units, starting 6-48 hours after injection with lethal doses of rickettsiae. (The unit age content of these samples of penicillin was determined by assay against *Staphylococcus aureus*.)

The rickettsiostatic and chemotherapeutic action of penicillin in eggs and mice was found in the same year to compare favorably with that of para-aminobenzoic acid.[2,3] Uniformly good rickettsiostasis by penicillin was noted in at least 5 different experiments.

In 1945 and 1946, the samples of penicillin which we obtained were usually completely ineffective against rickettsial infection in doses of 1000 *S. aureus* units per egg. Similar observations, indicating variability in the therapeutic action of commercial penicillin against bacterial and spirochetal infections, in spite of standardization *in vitro* in terms of bacteriostatic units, have been reported by others.[4] We considered the possibility that the satisfactory rickettsiostatic action of the earlier samples might have been caused by some impurity other than penicillin. The realization that commercial penicillin has contained varying amounts of several chemical types of penicillin, including penicillins G, K,

---

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Greiff, D., and Pinkerton, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 116.

[2] Moragues, V., Pinkerton, H., and Greiff, D., *J. Exp. Med.*, 1944, **79**, 431.

[3] Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

[4] *J.A.M.A.*, 1946, **131**, 271.

F and X[4] suggested another possible explanation of our inconstant results. The experiments to be reported here were planned to test the comparative rickettsiostatic action of these penicillin fractions.

Material and Methods. For the samples of penicillin, we are indebted to Dr. Leonard Karel of the National Institute of Health. The information furnished indicated that the purity of these compounds was relative rather than absolute. The penicillin G contained 1707 *S. aureus* units per mg and was estimated to be about 99.5% pure (chromatographic analysis with differential assay of various cuts). The penicillin K was really a mixture, containing 65% K, 18% dihydro-F, 7% F, and 7% G. The penicillin F was estimated by distribution curve to contain 6% of inactive material; otherwise the curve agreed well with the theoretical for penicillin F. The penicillin dihydro-F was said to contain 85% dihydro-F, 6% F, 7% K, and 2% G. The penicillin X by iodometric chemical assay gave figures indicating that it was about 92% pure; it was believed to contain no penicillins other than X; it was found to contain 918 *S. aureus* units per mg.

The samples used were not assayed in our laboratory for bacteriostatic action. From the work of others,[5,6,7] penicillin G, when assayed *in vitro* against *Staphylococcus aureus* or *Bacillus subtilis* is known to contain about 1667 units per mg (our sample 1707). Penicillin K contains (approximately) 2300 units per mg against *S. aureus* and 700 units per mg against *B. subtilis;* penicillin F 1500 units per mg against *S. aureus* and 970 against *B. subtilis;* penicillin X 900 units per mg against *S. aureus* (our sample 918) and 1500 against *B. subtilis.*[7] Because of this variable potency against different organisms, comparison of the samples for rickettsiostatic action was made primarily on a gravimetric basis.

Penicillin G (benzyl-penicillin) is presumably identical with the crystalline product designated penicillin II by British workers. Penicillin F (Penicillin I in British nomenclature) is Δ^2 pentenyl-penicillin, while penicillin dihydro-F is n-amyl-penicillin. Penicillin X (penicillin III in British nomenclature) is a p-hydroxy-benzylpenicillin. Penicillin K is n-heptyl-penicillin. The penicillin G was in the form of the potassium salt. All other samples used were in the form of sodium salts. It seems unnecessary to discuss the chemistry of the penicillins in detail, since no correlation between chemical structure and therapeutic action has been established.

A murine strain of typhus, maintained chiefly in fertile eggs for several years but recently passed through a guinea pig to establish its virulence, was used. The penicillin fractions were injected into the yolk sacs in two doses on the 2nd and 4th days after rickettsial injection. Groups of 24 to 36 eggs were used for each compound. The degree of infection in eggs dying or killed at various intervals was estimated by examining stained film preparations of the yolk sac membranes. Survival periods were also used as an index of chemotherapeutic action. The technics for this type of study have been described in detail in previous publications.[1,3]

The results of two separate experiments are shown in Tables I and II. In the experiment represented by Table I, the gravimetric dosage of penicillin X given was about twice as great as that of the other types, since we wished to make the unitage dosages approximately equal in terms of the activity of the compounds *in vitro* against *S. aureus.* This was done in order to facilitate comparison with samples of crude penicillin previously used. Table II represents an attempt to outline further the ranges of activity of the penicillin fractions. In a third experiment, the results of which are not given in tabular form, 1.2 mg of penicillin di-hydro F was found to have rickettsiostatic activity approximately equal to that shown by 0.6 mg of penicillin G. A more detailed assay of these compounds was not made because of insufficiency of the samples obtained.

---

[5] Williamson, J., and Lourie, E. M., *Brit. Med. J.*, 1946, June 1, page 829.

[6] Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, 1945, **49**, 411.

[7] Robertson, P., and Dufrenoy, J., *Bact. Rev.*, 1948, **12**, 79.

TABLE I.

Rickettsiostatic Action of Penicillins G, Di-hydro F, F, X, and K.

Age of embryo, days	Control	G 0.40 mg 666 units†	Di-F 0.40 mg	F 0.40 mg 666 units†	X 0.79 mg 666 units†	K 0.40 mg
5	Rickettsiae injected.					
7	333 units of penicillin injected.					
9	1112	112		23	00	1
9.5	333 units of penicillin injected.					
10	23344	11222	11222	2333	11	22333
		233	33	445		334
11	333333	111233	23333	33334	0* (+)*	33344
	44555		444	45	(+)* (+)	
13					0* (+)* 1	
14					0* (+)	
15					0* 1*	
16					0*	

Each figure represents an individual egg.
* Embryo alive at time of examination.
0 No rickettsia recognized.
(+) Less than one rickettsia per oil immersion field.
1 1-10 rickettsiae per oil immersion field.
2 10-100 rickettsiae per oil immersion field.
3 100-1000 rickettsiae per oil immersion field.
4 1000-5000 rickettsiae per oil immersion field.
5 5000-10,000 rickettsiae per oil immersion field
† Against *S. aureus* (estimated).

TABLE II.

Rickettsiostatic Action of Penicillins G, F, X, and K.

Age of embryo, days	Control	G 0.6 mg 1000 units†	G 1.2 mg 2000 units†	F 0.67 mg 1000 units†	X 0.60 mg 506 units†	K 0.60 mg
5	Rickettsiae injected.					
7	Penicillin injected.					
8		000	0 (+)	00 (+) 2	00	12
9	13	(+)	(+)	33	(+)	12233
9.5	Penicillin injected.					
10	35	(+) 111	0 (+) (+)	33333	(+) 111	3333
			(+) +	44445		45
11	444444	(+) 12222	0 (+) (+)	34444	11122	233333
	555555	33334	(+) (+)	44555		344444
	5555			55		55555
12	444444	(+) (+)	(+) (+)	3344	0 (+) (+)	(+) (+)
	444555	111222	(+) (+)	555	(+) (+)	11
	555		(+) (+)			222
			113			
13		0 (+) (+)	0 (+) (+)		0 (+)	
14			0* 0 (+)		0* 0*	
			(+) 1			
15			0* (+)*		0* 0* (+)*	
			(+) 11		(+) 2	
16					(+)* 2* 222	
17					0* 0* 0* 0*	

For explanation of symbols see footnote of Table I.

The results of these experiments suggest that penicillin X, on a gravimetric basis or on an estimated unitage basis against *B. subtilis*, may be about twice as active against typhus rickettsiae as penicillin G, while the other penicillins tested are much less potent. On an estimated unitage basis against *S. aureus*, penicillin X appeared to show about 4 times as much rickettsiostatic activity as penicillin G.

It is also worth noting that for the first time since 1944, effective rickettsiostasis was obtained by penicillin. Estimated doses of 1000 (*S. aureus*) units of penicillin G were definitely less effective than 1000 units of the crude penicillin mixtures used in 1944, but 2000 units of penicillin G and 506 units of penicillin X were about as effective as 1000 units of the crude mixture. Since the crude mixtures which we used in 1944 presumably did not contain significant amounts of penicillin X,[8] and probably did have fairly large proportions of penicillins other than G, the reason for their high effectiveness is not clear. It is conceivable that the penicillin fractions when mixed in certain proportions might have an effect greater than the sum of their individual effects.

It is now well known that the efficiency of the crystalline penicillins, as measured *in vitro,* varies with different bacteria and even with different strains of the same organism. Relationships found *in vitro* often have not held in experimental infection. Important factors *in vivo* seem to be the relative stability of the different molecular types, and their relative affinity for plasma proteins. Penicillin K has been shown to lose much of its potency because of the latter factor.[9] Rotman-Kauka and his co-workers[10] found that blood levels remained higher with penicillin X than with either commercial G or crystalline G, and that urinary excretion was higher for G than for X. In our experiments, levels of penicillin activity in the yolk sac were not determined. Loss by urinary excretion is largely eliminated by the use of the embryonate egg, but chemical disintegration and inactivation by protein absorption are both possible.

Penicillin X has been found to be much more active than the other types when tested *in vivo* against several bacterial infections. Penicillin G, however, has been found to be about 4 times as active as X against experimental syphilis[11] and experimental relapsing fever.[12] It may be noted that the results which we obtained in rickettsial infection are qualitatively and quantitatively similar to those obtained in experimental infection with several bacteria, and differ from those obtained with spirochaetal infections.

The figures given by Hobby and her co-workers[8] for example, against hemolytic streptococcus *in vivo* are X, 500; G, 100; F, less than 100; and K, 60 (on a unitary basis) and X, 270; G, 100; F, 92; and K, 82 (on a gravimetric basis). When tested *in vitro* against the same organism, all four of the samples which they used were about equally potent. It is also of interest that these workers found crude penicillin to be 3-5 times as effective *in vivo* as penicillin G and approximately equivalent to penicillin X. Here again their results parallel those which we have obtained against typhus rickettsiae. On the whole, the results which are reported here suggest that the samples of crude penicillin which we used in 1944 may have owed their effectiveness primarily to penicillin fractions. The possibility that impurities may have played a part directly, or indirectly by enhancing the action of penicillin has, however, not been ruled out. The possible nature and mode of action of such impurities has been discussed by others.[7]

As far as we are aware, adequate trials of penicillin in human rickettsial infection have not been made. Blake and his co-workers[13] have reported unencouraging results in two cases of tsutsugamuchi disease. One case (moderately severe) received about 266,000 units per day from the 4th to the 7th days of illness. This patient recovered, but there was no evidence that the penicillin altered the course of the illness. The second case received 120,000 units per day from the 5th to the 9th day of illness. This patient became critically ill on the 12th day of illness, but recovered eventually. Since the dosage which we found effective in mice translates to about 3,000,000 units per day for a patient weighing 150 lbs., and since as many as 10,000,000 units per day are at present used in subacute bacterial endocarditis, it is obvious that the trial of penicillin in these 2 cases had little value. Yeomans *et al.*[14] report the administration of penicillin to 4 cases of human typhus (dosages and other data not supplied) and were unable to decide whether or not the treatment was of value.

---

[8] Hobby, G. L., Burkhart, B., and Hyman, B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 296.

[9] Eagle, H., and Newman, E., *J. Clin. Invest.,* 1947, **26**, 903.

[10] Rotman-Kauka, G., Hirsh, H. I., and Dowling, H. F., *New Eng. J. Med.,* 1947, **236**, 314.

[11] Turner, T. B., Cumberland, M. C., and Huan-Ying Li, *Am. J. Syph., Gonor., and Ven. Dis.,* 1947, **31**, 476.

[12] Cumberland, M. C., and Turner, T. B., *Am. J. Syph., Gonor., and Ven. Dis.,* 1947, **31**, 485.

[13] Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.,* 1945, **41**, 243.

Several compounds having rickettsiostatic activity in animals have been tried thoroughly in human typhus infection, with definitely disappointing results.[15,16] In view of the relatively high toxicity of these compounds for man, and the very low toxicity of penicillin, it is rather surprising that adequate trials of the latter compound, using samples with experimentally proven potency, have not been made.

Summary and Conclusions. Samples of 5 relatively pure crystalline penicillin fractions have been tested for rickettsiostatic activity in typhus-infected embryonate eggs. Penicillin X appeared to be about twice as effective on a gravimetric basis, and about 4 times as effective on a (*S. aureus*) unitage basis as penicillin G. Penicillin F, di-hydro F, and K were much less effective. On a (*S. aureus*) unitage basis, penicillin X showed a potency approximately double that of samples of crude penicillin used in 1944. Penicillin G showed a very much higher potency (on a unitage basis) than most samples of partly purified penicillin which we tested in 1945 and 1946. The differential rickettsiostatic potency of these 5 penicillins is roughly parallel to their differential activity against certain bacteria *in vivo* as determined by other, but differs from that reported in experimental spirochaetal infections, in which penicillin G has been found most effective.

Irregular results obtained by us in the past with crude and partially purified commercial penicillins may perhaps be explained on the basis of varying proportions of penicillin fractions, although the possible existence of impurities having independent rickettsiostatic action or capable of enhancing the action of penicillin has not been ruled out. The factors of stability and protein absorption of the penicillins were not evaluated.

The effectiveness of penicillin in human rickettsial infection apparently remains undetermined. The only clinical trials in which data are available are invalidated by the use of low doses, started late in the course of the disease, by the small number of cases, and by the fact that samples of proven potency against the organism involved were not used.

---

[14] Yeomans, A., Snyder, J. C., Murray, E. C., Zarafonetis, C. J. D., and Ecke, R. S., *J. Am. Med. Assn.*, 1944, **126**, 349.

[15] Steele, J. M., McLimans, W. F., Grant, C. W., and Tullis, J. L., Report No. 6, January 14, 1946, Research Project X-222, Naval Medical Research Institute.

[16] Andrewes, C. H., King, H., van den Ende, M., and Walker, J., *Lancet*, 1944, **1**, 777.

16441

Distribution of Radioactive Carbon Administered as Carbonate in the Body and Excreta of Mature Rat.*

W. D. Armstrong, Jack Schubert, and Arthur Lindenbaum.
From the Department of Physiological Chemistry, University of Minnesota, Minneapolis, Minn.

Several studies have demonstrated the assimilation of carbon-dioxide by heterotropic organisms and mammalian tissue slices.[1] The intact animal also incorporates the carbon of carbon-dioxide as shown in experiments which utilized either the heavy (C^{13}) isotope,[2,3,4] or the short-lived radioactive (C^{11}) isotope[5] as tracers. Investigations with the intact animal have been limited by the difficulty of detecting C^{13} in high dilution or because of the rapid decay of C^{11}. It was found that the carbon of carbon-dioxide is incorporated in the liver glycogen,[3,5] bone,[4,5] urea,[2,4] and in certain amino acids of liver protein[4] of the rat. Further evidence for the uptake of the carbon of carbon-dioxide in bone was afforded[6] by radioautographs in experiments with rats using the long-lived C^{14} as a tracer. The radioautographs also indicated that C^{14} was present in the liver and kidney for periods up to 2 weeks.[6] We have investigated in further detail the quantitative distribution of C^{14} from carbon-dioxide in the intact animal and in the excreta. Because of the higher sensitivity of the methods for the determination of C^{14} over those of C^{13}, when the isotopes are present in high dilution, it has been possible to compare the assimilation of the labeled carbon in a greater number of tissues than has previously been possible. In this study the tracer isotope has been given parenterally as $Na_2C^{14}O_3$ and, as a means of prolonging the period of high C^{14} activity of the body fluids, as $CaC^{14}O_3$.

Experimental. Rat I—A male rat, weighing 624 g was given an intraperitoneal injection of 3.74 ml of a 0.043 molar solution of $Na_2C^{14}O_3$ (prepared by a method similar in principle to that of Allen, Gest, and Kamen)[7] and immediately placed in an apparatus (Fig. 1) which allowed the continuous collection of the exhaled carbon-dioxide and the separate collection of the urine and the feces for prolonged periods. At the end of 96, 120 and 144 hours additional 1.0 ml amounts of the $Na_2C^{14}O_3$ solution were administered by intraperitoneal injection. Each ml of the $Na_2C^{14}O_3$ solution contained about 0.1 millicurie C^{14} (2.05×10^6 counts per minute under our conditions of counting C^{14}). The collection of expired CO_2 was complete as indicated from experiments in which a known amount of $Na_2C^{14}O_3$ was placed in the apparatus and decomposed with acid with a resulting average recovery from 5 trials of $100 \pm 2\%$ of the theoretical $C^{14}O_2$. The diet, supplied without opening the apparatus, consisted of whole milk saturated with dextrose. The rat was accidentally killed by suffocation at about the 160th hour when its weight was 617 g.

Rat II—A male rat weighing 473 g was given about 0.40 millicurie of C^{14} (8.3×10^6 counts per minute) by implanting, through an abdominal incision, a pellet of $CaC^{14}O_3$

---

* This study was supported by a grant from the Research Grants Division of the U. S. Public Health Service. Some of the work described in this paper is to be submitted by Arthur Lindenbaum to the Graduate School of the University of Minnesota as a part of a thesis in partial fulfillment of the requirements for the Ph.D. Degree.

[1] Wood, H. G., *Physiol. Rev.*, 1946, **26**, 198.

[2] Swendseid, M. E., Ph.D. Thesis, Graduate School, University of Minnesota, 1941.

[3] Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, 1945, **159**, 475.

[4] Delluva, A. M., and Wilson, D. W., *J. Biol. Chem.*, 1946, **166**, 739.

[5] Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, 1941, **140**, 171.

[6] Bloom, W., Curtis, H. H., and McLean, F. C., *Science*, 1947, **105**, 45.

[7] Allen, M. B., Gest, H., and Kamen, M. D., *Arch. Biochem.*, 1947, **14**, 335.

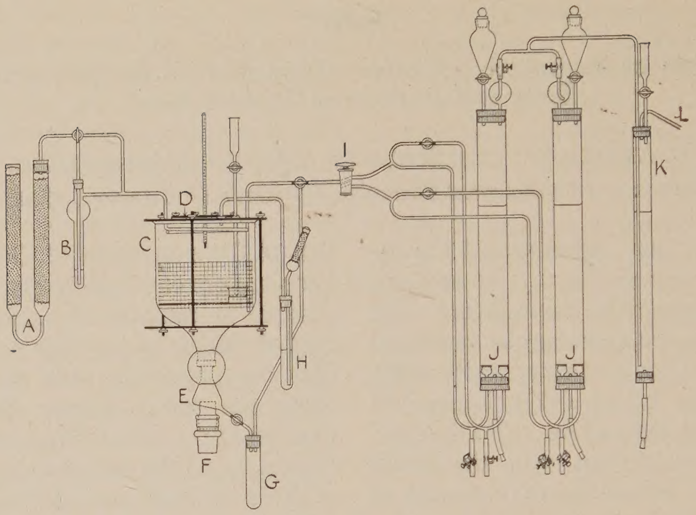


FIG. 1.

Apparatus for collection of expired carbon-dioxide and excreta of a rat. A, soda-lime tubes in air intake; B, bubble rate indicator which can be by-passed through 3-way stopcock shown; C, cage made from 10-liter bottle with bottom replaced with brass plate held as shown and sealed to glass with dentist's carding wax. Turbulent flow of gas in cage produced by air entering cage through multiple pin holes, directed upwards, in spiral tube near top of cage. Wire basket for support of animal covered with baked-on Glyptal varnish; D, port; E, urine and feces separator anchored to cage with plaster-of-paris; F, feces receptable; G, urine receptacle; H, auxiliary air intake; I, stopcock to direct exhaust air to one or the other CO_2 absorber; J, sintered glass disks in columns containing one liter 10% sodium hydroxide; K, trap; L, to suction.

TABLE I.

Comparison of C^{14} Assay by Direct Count of Sample with that Obtained by Wet Combustion and Subsequent Counting of $BaC^{14}O_3$.

		Specific activity† × 10^6	
Rat No.	Sample	Direct count	$BaCO_3$ from wet comb.
I	Glycerol	2.76 ± .088	2.81 ± .25
”	Feces (0-24 hr)	136 ± 1.1*	131 ± 2.6*
”	” (24-48 hr)	14.9 ± .16*	13.2 ± .81*
”	” (48-72 ”)	4.5 ± .23*	5.3 ± .39*
”	Defatted bone	19.5 ± .36	20.2 ± .89
”	Incisor dentin	34.0 ± .091	34.8 ± 1.1
II	Liver fatty acids	9.54 ± .15	10.8 ± 1.1
”	Protein—spleen	34.1 ± .34	34.0 ± .74
”	” —kidney	30.8 ± .33	28.0 ± .80
”	” —lung	21.7 ± .21	20.0 ± .80
”	” —heart	12.1 ± .39	11.2 ± 1.1
”	Liver glycogen	24.9 ± .28	26.4 ± .93
”	Plasma globulin	60.6 ± .65	61.8 ± 1.2

† % of injected dose per mg carbon.

* These results are given as the percentage of the total injected dose × 10^3.

in each of the lower quadrants of the peritoneal cavity. The pellets weighed 86.6 mg and 118.3 mg respectively, and were prepared from a mixture of 246 mg $CaC^{14}O_3$ and 10 mg gelatin suspended in warm distilled water. After the excess water was removed by slow evaporation, the $CaC^{14}O_3$-gelatin mixture was molded into pellets by manual manipulation

TABLE II.

Excretion of C^{14} by Rat I After Intraperitoneal Injections of C^{14} as Sodium Carbonate.

Injection No.	Time elapsed after first injection	% injected dose excreted			Specific activity $\times 10^6$†	
		Expired air*	Urine	Feces	Expired Air	Urine
1	0-1 hr	70.8	—	—	6.12×10^5	—
”	1-3 ”	21.4	—	—	22,100	—
”	3-6 ”	1.5	—	—	2,080	—
”	6-12 ”	0.68	—	—	405	—
”	12-24 ”	0.54	—	—	175	—
”	0-24 ”	94.65	0.45	0.31	15,400	2,710
”	24-48 ”	0.77	0.018	0.014	82.5	140
”	48-72 ”	0.21	0.011	0.0070	33.6	103
”	72-96 ”	0.13	0.0072	0.024	21.0	67.6
”	96 hr total	96.0	0.49	0.36	3,450	980
2	96-120 ” ”	103‡	0.090‡	0.15‡	17,800‡	536‡
3	120-144 ” ”	98.1‡	0.21‡	0.14‡	15,500‡	197‡
4	144-168 ” ”	91.2‡	0.071‡	0.26‡	25,800‡	518‡
—	0-168 ” ”	96.8	0.33	0.28	2,230	362

* The rat exhaled 560 ± 80 mM of CO_2 each 24 hr.

† Per cent of injected dose per milligram of carbon.

‡ These results represent the excretions of the C^{14} given in injections 2, 3, and 4 occurring during 24-hour periods following each administration. No correction was applied for the residual excretions of C^{14} from the previous injections.

TABLE III.

Excretion of C^{14} by Mature Rat II After Implantation of $CaC^{14}O_3$ in the Peritoneal Cavity.

Time elapsed after administration	% injected dose excreted			Specific activity* $\times 10^6$		Relative specific activity (Long bones = 100)	
	Expired air	Urine	Feces	Expired air	Urine	Expired air	Urine
2-21 hr	1.09	.0052	.00	255	39.0	480	73
21-45 ”	3.77	.0156	.0047	580	88.5	1,100	170
45-69 ”	9.42	.0414	.0052	1,380	212	2,600	400
69-93 ”	15.4	.0595	.0150	2,190	283	4,100	530
93-117 ”	20.8	.0802	.0253	2,830	374	5,300	700
117-141 ”	20.9	.0666	.0165	2,820	388	5,300	730
141-142 ”	0.80	—	—	2,650	—	5,000	—

* Per cent of injected dose per mg carbon.

while wearing rubber gloves and a surgeon's mask. The pellets were then dried in an oven. Collections of excreta and expired air were made, beginning 2 hours after the operation, over the time intervals indicated in Table III. Six days after the implantation of the pellets the animal was sacrificed by slitting its throat. A roentgenogram made at this time showed only a faint image of the disintegrated pellets. The weight of the animal just before being sacrificed was 474 g.

The blood was collected in a centrifuge tube containing heparin. Hemin was prepared by the method of Shemin and Rittenberg.[8] The solution remaining from the hemin isolation was treated with 10% sodium tungstate. The precipitate, which is presumed to consist mainly of globin, was washed with water, dried and pulverized. Albumin and globulin were isolated from the plasma by the method of Koch[9] and each protein was washed with trichloracetic acid solution.

The isolation and purification of liver glycogen, after removal of a sample of the organ for total C^{14} determination, and of muscle glycogen was carried out by the method of Stetten and Boxer.[10] Myosin was prepared from skeletal muscle using the pro-

---

[8] Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1946, **166**, 621.

[9] Koch, F. C., *Practical Methods in Biochemistry*, William Wood, Baltimore, Second Edition, 1937, p. 53.

[10] Stetten, G. E., and Boxer, D., Jr., *J. Biol. Chem.*, 1944, **155**, 231.

TABLE IV.

Distribution of C^{14} in the Tissues and Their Components in Rat II After Implantation of $CaC^{14}O_3$ in the Peritoneal Cavity.

Sample description	% of total administered dose, $\times 10^3$	Specific activity† (SA), $\times 10^6$	Relative (SA) (long bones = 100)
Long bones (less marrow)	22.0	53.1 ± .72	100
Short bones	68.0	49.9 ± .54	94
Inorganic CO_2 (long bones)	—	428 ± 2.5	810
Inorganic CO_2 (short bones)	—	408 ± 2.4	770
Long bone protein	—	10.6 ± .64	20
Short bone protein	—	12.1 ± .21	23
Long bone marrow	—	52.0 ± 1.3	98
Molar teeth	0.37 ± .010	—	—
Incisor teeth	1.30 ± .032	—	—
Molar dentine	—	28.7 ± .81	54
Incisor dentine	—	28.1 ± .53	53
Inorganic CO_2 of incisor dentine	—	474 ± 12	890
Incisor enamel	—	54.2 ± 4.0	102
Whole blood*	11.2	16.8 ± 1.1	32
Hemin	—	3.1 ± .15	5.8
Red blood cell protein	—	4.06 ± .27	7.6
Plasma albumin	—	74.1 ± .48	140
Plasma globulin	—	60.6 ± .65	110
Liver (intact)	365	48.7 ± .54	92
Liver glycogen	—	24.9 ± .28	47
Liver fatty acids	—	9.54 ± .15	18
Muscle protein	62.9	6.93 ± .16	13
Muscle glycogen	—	6.8 ± .9	13
Myosin from muscle	—	5.47 ± .17	10
Body fat	30.5	1.73 ± .11	3.3
Fatty acids from body fat	—	1.27 ± .090	2.4
Glycerol from body fat	—	10.1 ± .20	19
Fat from viscera	—	2.13 ± .068	4.0
Fat from bones and teeth	—	1.70 ± .055	3.2
Protein—G.I. tract	25.3	37.1 ± .41	70
" —spleen	2.64	34.1 ± .34	64
" —testicles	3.97	27.5 ± .26	52
" —kidney	4.79	30.8 ± .33	58
" —lungs	3.28	21.7 ± .17	41
" —heart	1.72	12.1 ± .39	23
" —brain + spinal cord	—	6.39 ± .17	12
Brain + spinal cord (intact)	1.50	5.24 ± .15	9.8
Pelt	130	2.20 ± .22	4.1
Debris + pellet remains	34,600	—	—

* The hemoglobin concentration was 14.9%.

† Per cent of injected dose per mg carbon.

cedure of Greenstein and Edsall.[11] The precipitate of myosin was extracted with alcohol and ether before being dried at 110°C.

Large samples of body and mesenteric fat were obtained by dissection and were separately processed. After the fats had been separated from non-lipid material by extraction in a Soxhlet apparatus with an alcohol-ether mixture they were dissolved in ether and washed several times with 5% $NaHCO_3$ solution and with water. The ether solutions were dried by the addition of anhydrous Na_2SO_4. After several hours the solutions were filtered and the ether removed by evaporation.

Aliquots of the fat samples were saponified with 40% NaOH and the resulting fatty acids and glycerol separated.[12] The fatty acids were freed of cholesterol and other non-saponifiable ether soluble substances by extracting a solution of the fatty acids in dilute

---

[11] Greenstein. J. P., and Edsall, J. T., *J. Biol Chem.*, 1940, **133**, 397.

[12] Koch, F. C., *Practical Methods in Biochemistry*, William Wood, Baltimore, Second Edition, 1937, pp. 25-26.

NaOH with ether. The free fatty acids resulting from acidification of the NaOH solution were washed 4 times with boiling distilled water, dissolved in ether and treated with active charcoal. The solution was filtered, dried with anhydrous Na_2SO_4 and the ether removed as previously described. A sample of liver fatty acids was prepared in the same manner from an ether extract of the acidified residue of this organ remaining from the isolation of the liver glycogen. The glycerol fractions were further purified as follows: An aqueous solution was extracted with ether, treated with active charcoal, filtered and concentrated on a hot plate. The resulting glycerol was water clear.

The proteins from each of several of the visceral organs, the testes and the central nervous system were prepared by suspending the pulverized, fat-free organ in 40% trichloracetic acid, centrifuging and subsequently extracting the precipitates with 10% trichloracetic acid. The precipitates were washed with water, alcohol and ether.

After skinning, the carcass was boiled in slightly alkaline water, permitting the bones and teeth and the skeletal muscles to be isolated. The bones and the teeth were dried at 110°C and extracted in a Soxhlet apparatus for 72 hours with an ether-alcohol mixture and dried at 110°C. The skeleton was divided into the *long* bones which consisted of the humeri, femora, tibiae, fibulae, radii and ulnae, and the *short* bones which comprised the rest of the skeleton. The marrow was removed from the long bones after they had been rendered fat-free and both fractions of the skeleton were finely pulverized. The preparations of bone protein consisted of the insoluble material obtained by digesting a part of each of the skeleton fractions in 40% trichloracetic acid, centrifuging, and subsequent washing of the precipitates with 10% trichloracetic acid, hydrochloric acid, water, alcohol, acetone and ether. The enamel and dentin of the molar and incisor teeth were separated[13] after the activity count of the whole teeth in the pulverized state had been made.

The pelt was digested with hot 20% sodium hydroxide, homogenized in a Waring blendor, evaporated to dryness and pulverized. All solutions resulting from processing the tissues, including the liver, and the organs and tissues not mentioned above were pooled to give a sample labeled "Debris." This mixture was evaporated to dryness and pulverized.

While dissecting the rat several small particles which apparently consisted of unabsorbed $CaC^{14}O_3$ were found. Their C^{14} content, present as inorganic carbon, was found to be 12.9% of the total administered dose. The fat-free residue from the mesenteric fat depot contained an amount of C^{14}, as inorganic carbon, which accounted for about 20% of the total dose of C^{14}. The fact that approximately 30% of the administered $CaC^{14}O_3$ remained unabsorbed does not alter the values for the relative specific activity of the samples given in Tables III and IV.

Rat III—About 145 mg of powdered $CaC^{14}O_3$ (0.065 millicurie or 1.3×10^6 counts per minute) were distributed over the peritoneal viscera of a 422 g male rat. Collections of expired air were made at intervals over a period of 40 days following the implantation of the $CaC^{14}O_3$. After sacrifice of the animal the carcass, less pelt, was ground in a meat chopper and homogenized with a small quantity of water in a Waring blendor, dried at 110°C and pulverized. The pelt was prepared for activity counts as previously described.

Method of Assay for C^{14} and Total Carbon Content. All samples were assayed for C^{14} using the technic described by Armstrong and Schubert.[14] When required the samples were subjected to wet combustion by adapting the method of Van Slyke and Folch[15] to a simple and rapid gravimetric procedure.[16] The evolved CO_2 was collected in a saturated solution of barium hydroxide and the resulting precipitate of $BaC^{14}O_3$ was washed and transferred to the brass filter dishes for weighing and counting. All samples of the $BaC^{14}O_3$ were stored in a desiccator until counted to prevent exchange with atmospheric carbon-dioxide.[17] The inorganic carbon of bone, dentin and enamel was determined and prepared for counting by collecting, in barium hydroxide, the CO_2 evolved from treatment of an aliquot of the pulverized material with 3 M perchloric acid.

---

[13] Manly, R. S., and Hodge, H. C., *J. Dental Research*, 1939, **18**, 133.

[14] Armstrong, W. D., and Schubert, J., *Anal. Chem.*, 1948, **20**, 270.

[15] Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, 1940, **136**, 509.

[16] Lindenbaum, A., Schubert, J., and Armstrong, W. D., *Anal. Chem.*, in press.

Several materials were counted directly as thick samples without combustion. Some of the samples which were counted directly were also oxidized, as described above, and the $C^{14}O_2$ collected and counted as $BaC^{14}O_3$. The agreement between the direct counts and the counts of $BaC^{14}O_3$ was good (Table I). When samples contained a higher percentage of carbon than is present in $BaCO_3$ it was advantageous to make a direct count because the activity was proportionately greater.[†]

Preparation of Radioautographs. A fat-free humerus and lower incisor from Rat I were dried, and attached to large corks, with a viscous mixture of acetone and "Duco" household cement. When dry the specimens were ground on a fine-grained carborundum stone until a plane section, about midway through the marrow or pulp cavity was obtained. The marrow or pulp cavity was then filled with the cement and the specimen was reground. The ground surface of the bone or tooth was placed in direct contact with Eastman No-Screen X-ray film in a light-tight container. At the end of two weeks the films were developed. Samples of bone from an untreated rat, when prepared and exposed in an identical manner, gave no image on the film. A kidney of Rat II was cut through the plane of the long axis of the hilum and the flat surface was kept in contact with the film for 2 weeks at 10°C.

Results and Comment. The deviations of the results given in the tables are derived from the statistical error of the radioactivity measurements.[‡] The data given in Tables II, III and IV summarize the findings regarding the excretion of C^{14} and the incorporation of this isotope in the tissues and derived components of Rats I and II.

Within 6 hours following the injection of $Na_2C^{14}O_3$ about 95% of the total injected dose of C^{14} appeared in the expired CO_2 (Table II). This finding agrees with that of Swendseid,[2] who injected mice with $NaHC^{13}O_3$ and followed their respiratory output of $C^{13}O_2$ for 3 to 4 hours. A sharp decrease followed the initial rapid rate of excretion of $C^{14}O_2$ and the rate became very much reduced about the 3rd or 4th day (Rat I). The results with regard to expired air can be taken to indicate that the $C^{14}O_2$ excreted after the first 24-48 hours, was, in large part, derived from C^{14} which had been fixed for a time in the animal's body. These results are of interest in regard to the health hazards associated with work with C^{14} since they indicate the probability of rapid excretion of a major part of the $C^{14}O_2$ inhaled during short exposures to this gas. Of the C^{14} given Rat I, 98% was recovered and 0.45% of the administered isotope was found in the animal at the time of death.[§]

The administration of C^{14} as pellets of

---

[17] Armstrong, W. D., and Schubert, J., *Science*, 1947, **106**, 403.

[†] Dr. Peter Yankwich (University of California) has indicated in a personal communication that the scattering of the beta-rays of C^{14} by $BaCO_3$ is somewhat higher than by organic biological materials. The agreement which we have observed between results calculated from counts made of unoxidized organic materials and of $BaC^{14}O_3$ derived from the same samples would, therefore, not be expected under all conditions of counting C^{14} in solid samples. It is suggested that a comparison of direct counts with those of $BaC^{14}O_3$ be made with the specific arrangement of counter and sample to be employed before direct C^{14} assays of biological materials are adopted as a routine procedure.

[‡] Square root of the sum of the squares of the standard deviations of the sample and background.

[§] The specific activities of the carbon and the fraction of the total dose of C^{14} present in the tissues and certain derived components of the tissues of Rat I were determined by methods essentially like those employed with Rat II but are not reported in detail. These data will be supplied upon request.

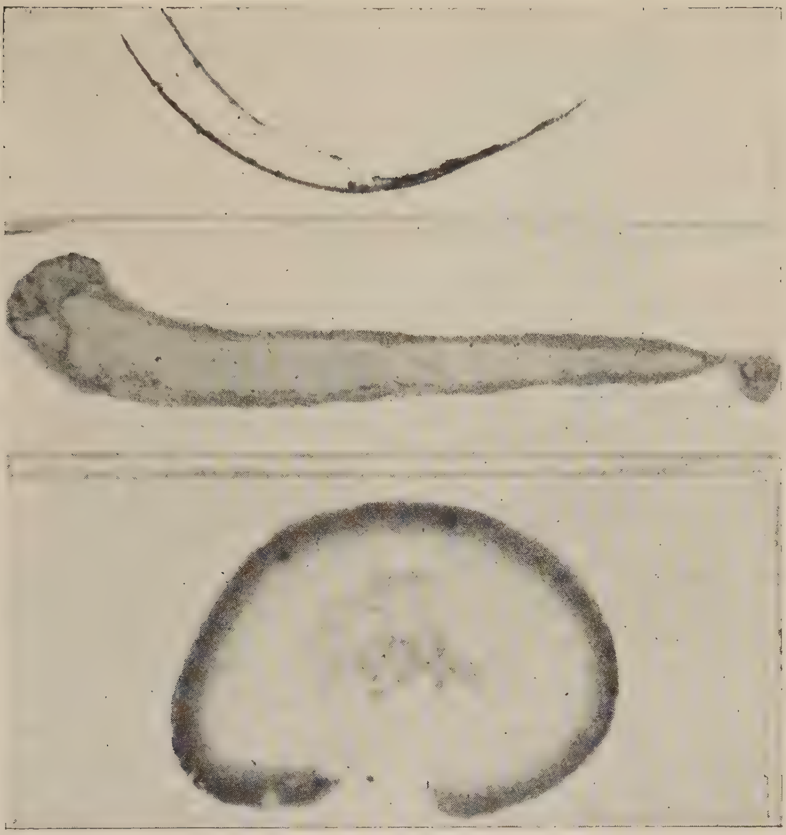


FIG. 2.
Radioautographs of a lower incisor (top), humerus (middle), and kidney (bottom) × 3.

$CaC^{14}O_3$ prolonged the period of elimination of the labeled carbon and resulted in the body fluids having a high and constant C^{14} specific activity for the 48-hour period preceding the sacrifice of Rat II (Table III). The largest amount of the administered C^{14} was present in the expired air of Rat III on the 7th-8th day and no detectable C^{14} was present in the expired air of this animal on the 22nd day following the implantation of $CaC^{14}O_3$. The C^{14} retained by Rat III, 40 days after administration, was $0.38 \pm .05\%$ of the total administered dose, $0.21 \pm .03\%$ being present in the pelt. These results indicate that a large fraction of C^{14} is excreted even when the isotope is present in the body as an insoluble inorganic compound.

In Rat II about 0.75% of the administered dose or about 1.0% of the adsorbed dose, was retained by the tissues. The C^{14} recovered in the tissues, excreta and expired air of Rat II was 108% of the administered amount. This result as to material balance is not in as good agreement with theory as was obtained with Rat I because of the uncertainties in the determination of the unabsorbed C^{14} from the pellets of $CaC^{14}O_3$.

It is apparent that the greatest incorporation of C^{14} in bone and teeth took place in the inorganic carbonate fraction. This turnover of carbon probably occurred mainly by exchange of carbonate groups of the mineral phase for labeled carbonate groups. The finding of C^{14} in the bone protein is of special significance since it is evidence, independent of the process of exchange of ions by the mineral phase, that bone tissue of a mature animal is continuously reformed. The C^{14} content of the inorganic carbon of bone and of the bone protein accounted for nearly 90%

of the total activity present in the skeleton of Rat I. There is a possibility that the bulk of the residual C^{14} activity may reside in the citric acid component of the skeleton since it is known that about 1% by weight of the dry fat-free bone is citric acid.[18]

The radioautograph of the incisor tooth of Rat I (Fig. 2) shows that the C^{14} was concentrated in a layer near to, or immediately adjacent to, the pulp cavity. Since this region is the location of the most recently formed dentin, the heavy concentration of C^{14} in this position is accounted for by deposition in the mineral phase of the dentin formed during the course of the experiment. The radioautograph of the kidney of Rat II shows that the C^{14} was concentrated in the cortical region.

The greater specific activity of C^{14} in the enamel of the incisor teeth than in the dentin (Rat II, Table IV) is probably due to the presence in enamel of a greater amount of inorganic carbon, which has a high uptake of C^{14}, than is present in dentin. No results as to the C^{14} content of the enamel of molar teeth were obtained because of poor yields of enamel from these teeth.

In the fat the greater part of the activity resides in the glycerol fraction but a definite incorporation of C^{14} in the fatty acids took place. The marked incorporation of C^{14} in the protein of the visceral organs is of interest. The relative amount of C^{14} incorporated by these proteins is in general agreement with their relative metabolic activity as determined by experiments with isotopic nitrogen.[19]

Other workers[2,5] were unable, in short term experiments, to demonstrate the incorporation of isotopic carbon (C^{11} or C^{13}), administered as inorganic carbon, in the muscle and liver glycogen of unfasted animals. Our results (Table IV) show, however, that such incorporation can be detected when the study is conducted under conditions which maintain the isotopic inorganic carbon content of the body fluids at a high level over a longer time.

Summary. Radioactive carbon was administered to unfasted mature rats either by intraperitoneal injection of $Na_2C^{14}O_3$ or by implantation in the peritoneal cavity of $CaC^{14}O_3$. Quantitative studies were made of the relative amounts of C^{14} excreted in the urine, the feces, expired air and incorporated by the tissues and derived components. A significant incorporation of C^{14} was found in the inorganic carbonate fraction of bone and in bone protein, the dentin, and enamel of the teeth, fatty acids, glycerol, hemin, red cell protein, plasma proteins, central nervous system protein, liver and muscle glycogen, muscle protein, and in the proteins of the testes and of the thoracic and abdominal viscera.

---

[18] Dickens, F., *Biochem. J.*, 1941, **35**, 1011.

[19] Schoenheimer, R., *The Dynamic State of Body Constituents*, Harvard University Press, Cambridge, Mass., 1942.

16442

Fractionation of Serum Cholesterol.*

J. C. Forbes, G. H. L. Dillard, W. B. Porter, and O. Petterson.
From the Departments of Biochemistry and Medicine, Medical College of Virginia, Richmond, Va.

During the last few years considerable evidence has accumulated indicating that the cholesterol of normal plasma is, to a very large extent, bound with the plasma proteins. Since cholesterol bound with plasma proteins would be completely soluble in the plasma, it is possible that its physiological behavior might be quite different from that of cholesterol which exists in the plasma unbound with

---

* We are indebted to Charles C. Haskell & Co., Richmond, Va., for financial support of this investigation.

TABLE I.
Serum Cholesterol Fractionation.

Subject	Age and sex, yr	Total cholesterol, mg %	Free chol., mg %	Readily extractable cholesterol, mg %	Neutral fat + cholesterol, mg %	Diagnosis
D.M.	50 M	197	97	33	—	Normal subject
C.H.	29 M	307	92	26	511	” ”
H.W.	24 M	276	78	28	440	” ”
M.U.	22 F	267	70	26	315	” ”
B.P.	25 F	229	43	19	363	” ”
A.B.	39 M	297	69	23	482	” ”
H.	28 M	170	32	12	256	” ”
R.E.	24 M	246	81	20	529	” ”
H.E.*	3 M	966	427	891	—	Nephrosis
		584	318	512	1664	”
		584	367	505	1635	”
A.J.*	38 F	585	198	484	—	”
		582	208	387		
W.E.	4 M	658	247	583	—	”
R.W.*	3 M	760	226	742	1768	”
		596	138	576	1376	
O.J.*	24 M	694	302	654	1897	”
		767	357	693	1682	
F.P.	4 M	1100	527	1070	—	”
B.H.	2 M	748	288	634	—	”
H.R.	42 M	1020	405	927	1397	Nephritis with nephrotic syndrome and diabetes
		817	268	728	1040	After a fat-free diet for 9 days

* The different determinations were run several weeks apart.

protein. The immediate purpose of the present investigation was to develop a method which might give the concentration of the *unbound* fraction. We have found that when normal serum is dried *in vacuo* from a frozen state, a relatively *small but constant* fraction of the total cholesterol of the dried serum can be extracted with chloroform at a temperature of about 5°C. This fraction will be referred to as the "readily extractable cholesterol." We postulate that it represents cholesterol which is either not bound with protein or held in very loose union.

Experimental. One-half cubic centimeter of serum was transferred to a half-ounce thick-walled screw-capped bottle. The serum was rapidly frozen and dried for an hour and a half at a pressure of about 0.2 mm. Immediately after drying, the bottles were put in a desiccator over calcium chloride. Shortly before extraction, the bottles were tightly capped with a screw-cap lined with tin foil, and put in a cold room at a temperature of around 5°C. When the temperature of the bottles had dropped to that of the room, 10 cc of cold chloroform were added to each, the bottles recapped and shaken at intervals thereafter. A 3-hour extraction period was adequate for maximum extraction of normal serum but as a special precaution we always extracted different samples of the same serum for both 3 and 24 hours. At the conclusion of the extraction period, the extracts were filtered cold, taken to the laboratory and analyzed for cholesterol, using a previously described procedure.[1] Inasmuch as the readily extractable cholesterol fraction, even in normal serum, contains both free and ester cholesterol, the recorded values are slightly higher than the true cholesterol value since cholesterol esters of the long-chain fatty acids give more color with the Liebermann-Burchard reagents than does an equivalent amount of cholesterol. The free and total cholesterol content of the undried serum was determined, using the procedure of Gershberg and Forbes.[1] Since the readily extractable

---

[1] Gershberg, H., and Forbes, J. C., *J. Lab. Clin. Med.*, 1942, **27**, 1439.

TABLE II.
Serum Cholesterol Fractionation—Rabbits on a High Cholesterol Diet.

Rabbit No.	Total chol., mg %	Readily extractable chol., mg %	Neutral fat + chol., mg %	Remarks
1-12	41* (22-69)	9* (5-15)	—	Normal rabbits
13	1373	1115	—	31 days on fat + cholesterol diet
	2690	2661	4834	45 ” ” ” ” ” ”
	1883	1712	3407	56 ” ” ” ” ” ”
	2373	2127	3157	77 ” ” ” ” ” ”
14	3167	2698	—	31 ” ” ” ” ” ”
	1391	1081	2277	45 ” ” ” ” ” ”
	2916	2863	3942	56 ” ” ” ” ” ”
15	2076	1873	—	31 ” ” ” ” ” ”
	2316	2325	4000	56 ” ” ” ” ” ”
	1850	1769	3448	71 ” ” ” ” ” ”
16	1142	1046	1540	38 ” ” ” ” ” ”
	3174	3158	6064	71 ” ” ” ” ” ”
17	2878	2880	5583	38 ” ” ” ” ” ”
	1679	1664	2556	71 ” ” ” ” ” ”

* Average of 12 rabbits, range in parentheses.

cholesterol fraction was not hydrolyzed prior to color development, the values for serum total cholesterol recorded in the tables also are given as the value obtained prior to hydrolysis of the cholesterol esters. The fraction designated as "neutral fat plus cholesterol" consists of neutral fat, free and ester cholesterol. A chloroform extract of undried serum obtained by a method previously described[2] was used for the analysis. The serum is adsorbed on an artificial zeolite (Doucil) which allows complete extraction of neutral fat and cholesterol by chloroform, but prevents extraction of the phospholipids. After filtration, an aliquot of the chloroform extract was evaporated to dryness and its neutral fat and cholesterol content determined by Bloor's oxidation method.[3]

Typical experimental results obtained on normal subjects and certain hospital patients are shown in Table I. Twenty-three normal individuals have been studied altogether. In these the readily extractable cholesterol fraction varied from 12-37 mg per 100 cc of serum, while 21 of the subjects gave values below 30 mg. The blood samples were usually taken 2 to 3 hours after breakfast. It is possible that the values might have been a little lower in the normal subjects if fasting blood samples were used. Eight nephrotic patients have been studied and the readily extractable cholesterol fraction was very high in all, being in some cases over 90% of the total serum cholesterol. Two cases of definite hypothyroidism gave elevated values for the readily extractable fraction, 49 and 58 mg, respectively. Two other subjects who showed no evidence of hypothyroidism except a somewhat elevated total cholesterol and a slightly subnormal B.M.R. gave values slightly above that of our highest normal value, the average of several determinations in each of these individuals being 40 and 43 mg. Fifteen diabetic patients have been studied. The readily extractable cholesterol fraction was quite high in one, slightly elevated in 4 and normal in 10. The results so far obtained do not allow any definite conclusion as to whether diabetic patients on the whole tend to have an elevation of this fraction or not. It should be noted that the diabetes was reasonably well controlled in all cases at the time the subjects were studied.

---

[2] Forbes, J. C., and Irving, H., *J. Lab. Clin. Med.*, 1931, **16**, 520.

[3] Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.

This problem is being studied in greater detail, with a particular attempt being made to correlate the laboratory data with the clinical findings.

Typical results obtained on a total of 20 hypercholesterolemic rabbits are shown in Table II. The experimental animals were fed Rockland rabbit pellets to which 5 g of cholesterol and 50 g of a hydrogenated vegetable oil (Mazola oil) dissolved in chloroform were added per kg, the whole thoroughly mixed and the chloroform allowed to evaporate. Blood was obtained by cardiac puncture at intervals and analyzed. It will be seen that the readily extractable fraction was very high in all cases, apparently comprising all of the cholesterol of the serum in some cases. All of the experimental animals at the time of autopsy showed some atherosclerotic lesions. Animals fed a similar diet but without added fat showed the same general picture except for a considerably greater fluctuation in the different cholesterol fractions amongst the individual animals.

Discussion. Although the significance of the variations in the concentration of the readily extractable cholesterol of the serum remains to be determined, it is of considerable interest that the two conditions in which it was found to be exceptionally high, namely clinical nephrosis and experimental hypercholesterolemia of rabbits, both tend toward the rather rapid development of atherosclerotic lesions. More extensive studies, however, are necessary before any conclusions regarding its significance can be drawn.

Summary. Chloroform extraction of normal serum, dried *in vacuo* from a frozen state, removed a relatively small but constant fraction of the total cholesterol. A very high percentage of the total cholesterol of the serum of nephrotic patients was extracted under the same conditions. The same was true of rabbits rendered hypercholesterolemic by the administration of cholesterol with their food. The readily extractable fraction was slightly elevated in all the hypothyroid cases studied and in some diabetic patients.

16443

Disappearance of Cholesterol Following its Intravenous Injection in Physiologically Emulsified Form.*

L. Horlick,[†] M. Feldman, Jr., and L. N. Katz.

From the Cardiovascular Department,[‡] Medical Research Institute, Michael Reese Hospital, Chicago.

Despite the implication of cholesterol in many vital processes, such as in the formation of hormones and in the genesis of atherosclerosis, very little is known concerning its metabolism. As technics in metabolic studies oral tolerance tests have consistently failed because of the slow and irregular absorption of cholesterol suspensions from the gastrointestinal tract, and the resultant insignificant rise in blood levels.[1-4] Artificial emulsions prepared with high pressure emulsifiers and various emulsifying agents, such as the lecithins and phospholipids have been unsatisfactory, either because the particle size obtained was too large for safe intravenous administration,

---

[*] Aided by a grant from the Life Insurance Medical Research Fund.

[†] Dazian Fellow.

[‡] The Department is supported in part by the Michael Reese Research Foundation.

[1] Brun, G., Changes in the Lipid Contents of Serum in Patients with Manic-Depressive Psychosis, H. K. Lewis, London, 1940.

[2] Wendt, H., *Biochem. Z.*, 1932, **250**, 212.

[3] Gardner, J. A., and Gainsborough, H., *Biochem. J.*, 1928, **22**, 1048.

[4] Turner, K. B., and Steiner, A., *J. Clin. Invest.*, 1939, **18**, 45.

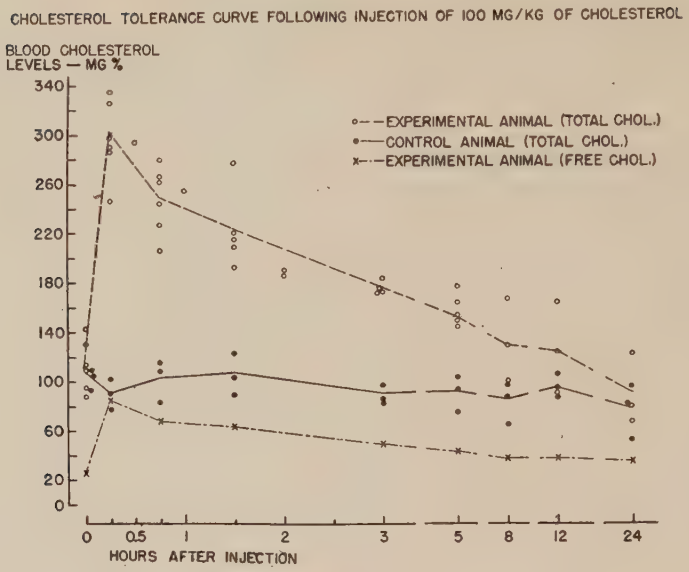


Fig. 1.

or because the concentration of cholesterol in the emulsion was too low.[5]

We have observed that chickens placed on a high cholesterol diet develop a hyperlipemia and hypercholesterolemia, with blood cholesterol levels frequently exceeding 2000 mg %.[6] It occurred to us that plasma from such blood could be employed in the study of cholesterol metabolism in the chicken. Relatively small volumes of the plasma would provide relatively large doses of cholesterol.

White leghorn chickens were fed a high cholesterol diet (4%) for at least 3 weeks, and then bled of 20 cc each. The heparinized blood was centrifuged, the milky plasma drawn off and passed through a Seitz filter in order to assure sterility. It was stored in the refrigerator until required. Plasma cholesterol determinations by the Schoenheimer-Sperry method[7] showed no change before or after filtration and storage for periods up to 10 days.

Tests of cholesterol disappearance were performed on normal adult white leghorn cockerels given the stored plasma intravenously in doses providing 100, 150 and 200 mg of cholesterol/kilo of body weight (approximately 6 cc of injected plasma). The blood levels were followed at intervals. A series of control animals which did not receive the plasma injection were subjected to the same tests. The injection of the lipemic plasma produced no injuries or deaths, and repeated injections caused no anaphylactic reactions.

Fig. 1 summarizes the curves obtained on 6 normal adult birds injected with lipemic plasma, providing 100 mg of cholesterol per kilo. Fifteen minutes after injection the average blood cholesterol level had risen from the initial value of 114 mg % to 297 mg %, then it declined gradually to a level somewhat below normal in 24 hours. The free cholesterol paralleled the total cholesterol levels throughout. The slope of the individual curves resembled the slope of the average curve.

The cholesterol levels on 3 normal adult control chickens remained relatively unchanged during the 24-hour period. In 2 additional adult chickens injected with a dose of 200 mg of cholesterol per kilo, and 1 with 150 mg per kilo, the peak levels were higher but the slope of the curve paralleled closely the average curve obtained with 100 mg per

---

[5] Cole, W. H., Clark, H., and Womack, N. A., *J. Lab. Clin. Invest.*, 1941, **26**, 1679.

[6] Horlick, L., and Katz, L. N., in press.

[7] Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

TABLE I.
Total Circulating Blood Cholesterol Above Control Value.*

Time after injection	100 mg/kg dose	200 mg/kg dose
15 min	220 mg	498 mg
45 ”	160 ”	428 ”
90 ”	132 ”	350 ”
3 hr	65 ”	188 ”
5 ”	43 ”	221 ”
8 ”	20 ”	83 ”
12 ”	16 ”	58 ”
24 ”	—27 ”	—31.2 ”

* The circulating blood volume was assumed to be 120 cc. The values given are averages, those connected by the lines in Fig. 1 are means.

kilo. At 24 hours, the levels had returned to normal.

The data in Table I show the total amount of cholesterol in the circulating blood in excess of the control value at the various time periods. The values are based on the total blood volume, estimated in our laboratory by the Evans blue method to be 120 cc for the adult chicken. The rate of disappearance of the injected cholesterol varies with its concentration above the control value. Thus, when the difference is 220 mg a fall of 60 mg occurs in 30 minutes, but when the difference is only 20 mg the fall is 4 mg in 4 hours. When the excess of circulating blood cholesterol is plotted against time on a logarithmic scale, a straight line relationship obtains up to 8 hours following injection; between 8 and 12 hours there is a decline in the slope; and between 12 and 24 hours the original relationship again obtains. The same relationship holds also for free cholesterol.

Discussion. Following the injection of physiologically emulsified cholesterol into the chicken consistent cholesterol disappearance curves were obtained. The highest blood levels were observed 15 minutes after injection, after which the cholesterol level fell gradually. The return of the cholesterol from the peak to the control level in 12 to 24 hours is in marked contrast to the return of glucose to the normal control level in 2 to 3 hours. We have shown that the removal of cholesterol from the blood is a function of its concentration in the blood, and that at high blood concentrations, the organism can remove relatively large amounts of cholesterol from the blood in short periods of time. The method has also been applied successfully in the rabbit.

Our technic involves the introduction of all the elements of the lipemic plasma, neutral fats, fatty acids, phospholipids, cholesterol, plasma proteins, etc., which are probably finely adjusted physiologically to maintain the lipemic plasma cholesterol in its native emulsified state. We can therefore essentially duplicate the conditions imposed by high dietary loads of cholesterol and thus obtain a picture of how the organism deals with exogenous cholesterol. The method is thus superior on theoretical grounds to those using artificially prepared emulsions, which the organism presumably must convert into physiologically acceptable form prior to disposal.

Summary. 1. A technic for obtaining an intravenous cholesterol tolerance test in the chicken is described.

2. Cholesterol is removed from the blood stream rapidly when the blood cholesterol levels are high and slowly when the blood cholesterol levels are low.

3. Total cholesterol and free cholesterol levels parallel each other throughout such tests.

16444 P

Staphylococcal Fibrinolysis.*

E. B. Gerheim,† J. H. Ferguson, B. L. Travis, C. L. Johnston, and P. W. Boyles.

From the Department of Physiology, University of North Carolina, Chapel Hill.

The ability of certain pathogenic staphylococci to clot decalcified blood plasma (oxalated or citrated blood) depends upon a mechanism distinct from the activation of prothromblin.[1,2] A recent paper[3] describes the preparation and some properties of a stable staphylocoagulase, together with data to indicate that the co-factor ("activator substance"), which it needs in order to coagulate fibrinogen, can be provided by certain plasma protein fractions, particularly albumins. It seems logical to reserve the term "staphylocoagulase" for the active coagulant and to propose the term *prostaphylocoagulase* for the bacterial product. In tests reported in a previous abstract,[4] the clots formed by the action on *bovine* plasma Fraction-I (fibrinogen) of prostaphylocoagulase plus human serum (or plasma) albumin did not show fibrinolysis for as long as 10 days at 37°C. This is attributable to lack of precursor of the fibrinolytic protease in the bovine plasma product.[5] In the tests of the present communication, *human* plasma Fraction-I is chosen because of its content of pro-enzyme, demonstrated with the aid of streptokinase, as in the previous study.[5] The following experiments prove the ability of the staphylococcal product to act as a kinase activator of the plasma protease precursor and also introduce new evidence that the coagulative and lytic-aiding properties of staphylococci are separable phenomena.

Materials and methods. Previous papers[3,5] give details concerning the reagents: 1. borate buffer (buff.), here used throughout with the addition of 0.4% trisod. citrate (pH: 7.7); 2. H.S.A., human serum albumin; 3. B.F. bovine plasma fraction-I (fibrinogen); 4. H.F., human plasma fraction-I,‡ containing fibrinogen, protease precursor (tryptogen or plasminogen), and co-factor for staphylocoagulase; 5. Strep., streptokinase; 6. Staph. *pro*-staphylocoagulase. In order to secure rapid firm fibrin clots for timing of fibrinolysis, a little thrombin (thr.: the commercial preparation "thrombin topical," 10 units per cc) was added after the other reagents in these tests.

Data. Table I compares the fibrinolytic effects of similarly prepared (1%) extracts of streptokinase (*2*) and (pro)staphylocoagulase (*3*). The *staph.* preparation is considerably slower than the strep. (L.T.—6 min.), but produces complete fibrinolysis in 3 hours. A negligible protease contaminant of the H.F. or thr. (probably the latter) shows up in the control (*1*) as no lysis in 48 hours but about half the clot remaining at the end of 4 days (37°C).

The different thermostability characteristics of the coagulative and lytic effects of the staphylococcal product were studied by incubating a 1% staph. solution in citrated borate buffer and testing at intervals, either

---

* This is the seventh of a series of studies aided by a grant from the John and Mary R. Markle Foundation.

† Predoctorate Research Fellow, U. S. Public Health Service.

[1] Gratia, A., *C. R. Soc. Biol.*, 1919, **82**, 1245, 1247, 1393.

[2] Gratia, A., *ibid.*, 1920, **83**, 584, 585, 649, 1221.

[3] Gerheim, E. B., Ferguson, J. H., and Travis, B. L., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 525.

[4] Gerheim, E. B., Ferguson, J. H., and Travis, B. L., *Fed. Proc.*, 1948, **7**, 41.

[5] Ferguson, J. H., Travis, B. L., and Gerheim, E. B., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 285.

‡ The human plasma fraction used in this work was prepared from blood collected by the American Red Cross, under contract between the O.S.R.D. and Lederle Laboratories, and supplied through the courtesy of Dr. R. W. Howard, Mr. Frank H. Clarke, and colleagues.

TABLE I.
Effects of Streptokinase and Staphylokinase on Fibrinolysis.

	H.F., 1% 1 cc	Thr., 10u 0.5 cc	Strep., 1% 0.25 cc	Staph., 1% 0.25 cc	Buffer (citrated)	Clot-lysis at 37°C
1	+	+			+	∞ 2 d
2	+	+	+			6 min
3	+	+		+		3 hr

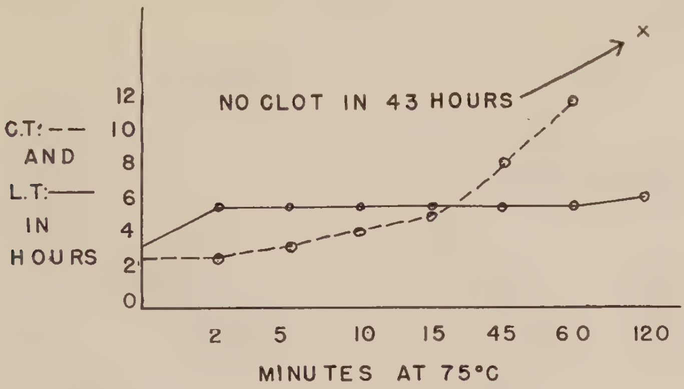


FIG. 1.
Thermostability study of prostaphylocoagulase and staphylokinase. 75°C. C.T.: staphylocoagulation times (at 37°C) for 1 cc B.F. + 0.25 cc staph. + 0.25 cc H.S.A. L.T.: fibrinolysis times (at 37°C) for 1 cc H.F. + 0.25 cc thr. + 0.25 cc staph.

on B.F. + H.S.A. (for staphylocoagulation), or on H.F. + thr. (for fibrinolysis). In a first experiment, at 57°C, the staphylocoagulation times (C.T.) lengthened from 55 minutes at the start to 370 minutes after a 6-hour heating period, whereas the fibrinolysis times (L.T.) remained about the same (3 hours). In a second experiment, the data of which are shown graphically in Fig. 1, incubation was at 75°C, and after 2 hours staphylocoagulation was lost (C.T. > 43 hours), while lysis times were relatively little altered (L.T.: 3-5½ hours).

Conclusions. The data clearly indicate the separability of the coagulative and lytic-aiding properties associated with staphylococci. Other current experiments suggest that the proportions of prostaphylocoagulase and staphylokinase in the alcohol-precipitated product vary with the strain of micro-organism. Since staphylocoagulation is produced by prostaphylocoagulase *plus* a plasma cofactor other than prothrombin (our citration technic gives extra assurance of this), the use of the term "staphylokinase" by Much,[6] to indicate an action like thrombokinase (or thromboplastin), is not correct. We propose the term *prostaphylocoagulase* for this factor, and retain the term *staphylokinase* for the second factor, which acts like streptokinase. In a previous publication[5] we cited recent work which proves that the streptococcal factor does not act as a proteolytic enzyme in its own right (*cf.*[7]), but merely serves as a kinase activator for the precursor of plasma protease (tryptogen or plasminogen). Similarly, the older work (*e.g.*[8]), attributing a protease to staphylococci, must be revised in the light of the data in this present study. We are obviously again dealing with a *tryptokinase*[9] or activator of plasma protease. It may be recalled that crystalline pancreatic trypsinogen is activated to trypsin by a mold kinase.[10]

---

[6] Much, H., *Biochem. Z.*, 1908, **14**, 143.

[7] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1934, **60**, 239.

[8] Fredericq, P., *Actualitéi biochimiques*, 1946, **5**, 1.

[9] Ferguson, J. H., *Quarterly Phi Beta Pi*, 1948, **44**, 279.

[10] Northrop, J. H., Kunitz, M., and Herriott, R. M., *Crystalline Enzymes*, Columbia Univ. Press, N. Y., 1948, 2nd ed., 352 pp.

Addendum. In a recent note (*Nature*, 1948, **161**, 559), published after our original communication[4] and received while the present article was in press, C. H. Lack reports confirmation of the staphylo*kinase* factor.

16445

Oleic Acid Toxicity and Fat Embolism.*

N. C. Jefferson and H. Necheles.

From the Department of Gastro-Intestinal Research, Medical Research Institute of Michael Reese Hospital, and the Department of Surgery, Provident Hospital, Chicago, Ill.

In view of the paucity of data on the toxicity of fatty acids, experiments with oleic acid were performed. In control tests, olive oil was injected intravenously, and observations were made pertaining to fat embolism.

The hemolytic effects of fatty acids are a well known phenomenon. This report deals with toxic manifestations observed in addition to those of hemolysis. The intravenous injection of fat emulsion was studied by a number of investigators such as Murlin and Riche;[1] Kochne and Mundel;[2] Gordon and Levine;[3] Clark and Brunschwig;[4] Dunham and Brunschwig;[5] McKibbin *et al.*;[6] Shafiroff and Frank;[7] LeVeen;[8] and reportedly by Russian workers who used human fat.

Johnson *et al.*[9–11] have demonstrated increased erythrocyte fragility after the ingestion of large fat meals, due to fatty acids and soaps which enter the blood with the lymph of the thoracic duct.

Dubois *et al.*[12] and Davis[13] demonstrated that serum albumin has considerable affinity for oleic acid, thereby inhibiting the bacteriostatic effects and toxicity of the latter.

For assays of toxicity, 36 normal, starved, unanesthetized, mongrel dogs were used. A few assays were performed on dogs anesthetized with pentobarbital sodium, in which carotid blood pressures were recorded.

The substances used were oleic acid (95% oleic acid and 5% other long chain fatty acids), ethyl, oleate, and sodium oleate; the latter 2 were prepared from the oleic acid. All substances were administered intravenously. The oleic acid and the ethyl oleate and valuable advice were obtained by the kindness of Dr. A. W. Ralston of the Armour Laboratories.

Results. Doses of 0.25 cc of oleic acid had little effect (6 dogs), while doses of 0.5 to 5 cc were toxic (19 dogs). The outstanding symptoms were: drop in blood pressure, urination and defecation, and rapid, forced, deep respirations, coughing, and development of massive pulmonary edema with large amounts of bloody, frothy exudate from the mouth and nose, followed by collapse and death. Life after injections of 5 cc lasted from 5 minutes to 2 hours, with an average of 40 minutes.

---

* Aided by a grant from the Otto Baer Fund. The Department is in part supported by the Michael Reese Research Foundation.

[1] Murlin, J. R., and Riche, J. A., Proc. Soc. Exp. Biol. and Med., 1915, **13**, 7.

[2] Kochne, M., and Mundel, L. B., *J. Nutr.*, 1929, **1**, 399.

[3] Gordon, H. H., and Levine, S. L., *Am. J. Dis. Child.*, 1935, **50**, 894.

[4] Clark, D. E., and Brunschwig, A., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 329.

[5] Dunham, L. J., and Brunschwig, A., *Arch. Surg.*, 1944, **48**, 395.

[6] McKibbin, J. M., Pope, A., Thayer, B. A., Ferry, R. M., and Stare, F. J., *J. Lab. Clin. Med.*, 1945, **30**, 485.

[7] Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

[8] LeVeen, H. H., *Fed. Proc.*, 1948, **7**, 277.

[9] Loewy, A., Freeman, L. W., Marchello, A., and Johnson, V., *Am. J. Physiol.*, 1942, **138**, 230.

[10] Longini, J., and Johnson, V., *Am. J. Physiol.*, 1943, **140**, 349.

[11] Freeman, L. W., Loewy, A., and Johnson, V., *Am. J. Physiol.*, 1944, **140**, 556.

[12] Davis, B. D., and Dubois, R. J., *J. Exp. Med.*, 1947, **86**, 215.

[13] Davis, B. D., *American Scientist*, Autumn issue, 1946.

Hemolysis occurred, varying in degree from 75 mg % of free hemoglobin to almost complete hemolysis (13.54 gm per 100 ml), depending on the dose of oleic acid given. In animals with low degrees of hemolysis, the hematocrit was elevated. Blood pH usually dropped to the acid side, from 7.46 to 6.37; the lowest value of 6.37 was obtained in a moribund animal, that died within a few minutes. If an animal survived for 1 hour or more, blood pH returned to normal or to alkaline values; the highest alkaline pH observed was 8.2. Serum calcium dropped to 6-7 mg % in most animals, and in 2 dogs to as low as 2 and 0.6 mg %; the latter values appeared in moribund animals that showed the highest degrees of hemolysis.

Autopsy showed the following: the gastrointestinal tract was markedly contracted, especially the pyloric antrum of the stomach; however, the cardia and fundus of the stomach were dilated. The urinary bladder was contracted maximally. The most significant changes were found in the chest. The thoracic cavities contained much bloody fluid. the lungs were edematous and hemorrhagic throughout, and on section bloody fluid oozed from the parenchyma and from the bronchi. The amount of hemorrhage and edema far exceeded any condition which might be ascribed to fat embolism.

Microscopically, in formalin fixed frozen sections, stained with scarlet red and hematoxylin eosin, red masses in the pulmonary vessels varying in size from specks to globules, massive edema of the lungs, and intestinal hemorrhages were observed. Heart, liver, kidneys, and adrenals, were found to contain fat staining material, however not to a strikingly larger extent than tissues from normal controls; that is lipids in the hepatic cells, in the bile duct cells and in the heart did not appear to be increased or altered by the injection of oleic acid.

Three dogs were injected repeatedly with sublethal doses of oleic acid. They presented the symptoms of increased and forced respiration as described above, but recovered within a day. They seemed to acquire a tolerance to oleic acid which manifested itself by decreasing hemolysis and, after 12 injections, practically no hemolysis was observed. The livers in these chronic dogs were pale yellow and friable, and showed microscopic evidence of fatty metamorphosis.

In order to analyze whether the toxic effect of oleic acid was due to the free carboxyl radical, ethyl oleate was injected in doses of 0.5 to 5.0 cc, (3 dogs). Ethyl oleate was toxic, showing the same clinical symptoms as oleic acid but no edema or hemorrhage of the lungs and no hemolysis, and it was never lethal. Animals sacrificed 1-2 hours after injection did not show the pulmonary changes seen with oleic acid, either grossly or microscopically.

Oleic acid forms soaps in an alkaline medium. In order to analyze the possibility that the toxic effects of oleic acid were due to this, sodium oleate (2 g) was injected (3 dogs). The animals died within a few minutes, showing nearly complete hemolysis.

Comparable amounts of pure olive oil (5 cc) injected intravenously gave no untoward reactions nor hemolysis (2 dogs). Microscopic sections revealed large amounts of fat-staining material in the capillaries of the lungs, much more than with any of the other materials studied, namely oleic acid, ethyl oleate and sodium oleate; yet olive oil was the least toxic of all substances tested. The dogs acted normally and did not seem to be affected in the least. The massiveness of fat embolism as seen microscopically after olive oil injection and the lack of symptoms do not seem to fit too well into the clinical picture of fat embolism.

Conclusions and summary. If, according to the findings of Davis and Dubois, albumin has the power of binding oleic acid in the ratio of 9 to 1, then the amounts of oleic acid injected exceeded the protective range in the dogs used in our experiments.

The mechanism of the toxic action of oleic acid is problematical. Small doses of sodium oleate produced a degree of hemolysis never seen with comparable doses of oleic acid.

While changes in blood serum calcium appeared to be correlated with the degree of hemolysis, the low values of serum calcium may be explained by formation of calcium soaps. Changes of blood pH appeared to be associated with respiratory conditions and with acid and toxic effects of the oleic acid.

Oleic acid does not seem to produce massive edema of the lungs by embolism, but by toxic effects on the capillaries of the lungs. Caution must be used in the preparation of fat emulsions for intravenous alimentation, in order to exclude fatty acids and soaps. Fat embolism *per se* may not be so dangerous unless fatty acids are released in or into the circulation.

16446

In vitro Effect of Streptomycin and Para-Aminosalicylic Acid (PAS) on the Growth of Tubercle Bacilli.*

KIRSTEN VENNESLAND, ROBERT H. EBERT, AND ROBERT G. BLOCH.

From the Department of Medicine, University of Chicago.

On the basis of Bernheim's[1] observations that the sodium salts of benzoic and salicylic acids specifically stimulated the oxygen uptake of the tubercle bacillus, Lehman[2,3,4] undertook to find a substance of similar chemical structure which might prove bacteriostatic for this organism. Of the 60 derivatives of benzoic acid studied, 4 amino-2-hydroxy benzoic acid (para-aminosalicylic acid or PAS) was found to be the most effective, inhibiting growth of the tubercle bacillus *in vitro* in concentrations as low as 0.15 mg %. This effect was found to be bacteriostatic rather than bacteriocidal. Sievers[5] and Youmans[6] soon confirmed these findings. Youmans reported that 6 streptomycin-resistant strains of tubercle bacilli tested by him were equally sensitive to PAS.

Smith and McClosky[7] and others have reported that a combination of two bacteriostatic agents often is more effective against the tubercle bacillus than either drug alone. It was logical to assume, therefore, that PAS plus streptomycin might behave in a similar manner; in this paper we report the *in vitro* effects on the tubercle bacillus of each of the 2 drugs alone and in combination.

While these experiments were in progress, Youmans[8] reported that the *in vitro* bacteriostatic effect of PAS in combination with streptomycin was no greater than the sum of the individual activities of the two substances.

Methods. Cultures were grown in the liquid medium described by Dubos,[9] and the concentration of tubercle bacilli in mg/cc was determined by turbidimetric readings in calibrated tubes using a blue filter (420 mμ) in a Lumetron colorimeter.

The following 2 test organisms were used: (A) the human strain of tubercle bacilli H-37RV, which is streptomycin-sensitive and (B) the naturally occurring strep-

---

* This work was sponsored by a grant from the United States Public Health Service.

[1] Bernheim, F., *J. Bact.*, 1941, **41**, 387.

[2] Lehman, J., *Lancet*, 1946, **1**, 14.

[3] Lehman, J., *Lancet*, 1946, **1**, 15.

[4] Lehman, J., *Nordisk Medicin*, 1947, **33**, 140.

[5] Sievers, O., *Nordisk Medicin*, 1947, **33**, 145.

[6] Youmans, G. P., *Quart. Bull. Northwestern Univ. Med. School*, 1946, **20**, 420.

[7] Smith, M. I., and McClosky, W. T., *Pub. Health Rep.*, 1945, **60**, 1129.

[8] Youmans, G. P., Raleigh, G. W., and Youmans, A. S., *J. Bact.*, 1947, **54**, 409.

[9] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

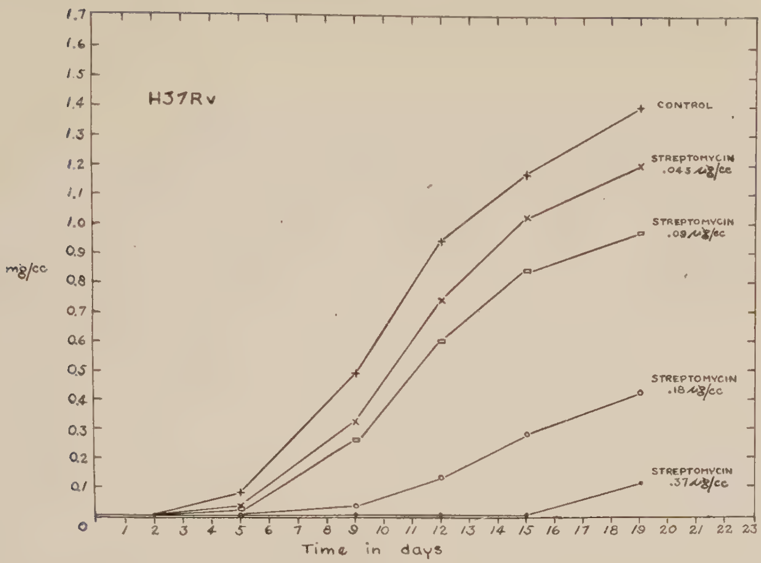


FIG. 1.

The effect of various concentrations of streptomycin on the growth of a virulent human strain of tubercle bacilli (H-37RV) charted in mg/cc of total growth at various time intervals. (0.74 mg of streptomycin per cc caused complete inhibition of growth and is not shown in the graph.)

tomycin-resistant variant H-37RVNR1.[10] Several other virulent strains of human tubercle bacilli isolated from sputum of patients with pulmonary tuberculosis were tested and behaved in a similar manner. Stock cultures of all strains were kept in Dubos liquid medium and were subcultured at 7- to 14-day intervals.

Commercial streptomycin sulfate was used, and dilutions were made aseptically from a stock solution prepared by adding 10 cc of sterile distilled water to 1 g of the powdered drug. The stock solution was freshly prepared for each experiment.

The PAS, which was supplied by Parke Davis in powdered form, was not sterile. It was accurately weighed for each experiment, dissolved in distilled water or Dubos medium, and sterilized by passing it through a Seitz filter. PAS is unstable at high temperatures and loses some activity if sterilized by heat.[11]

In each experiment a measured inoculum of 0.1 mg of tubercle bacilli per tube was used and the same stock culture was used as the source of the inoculum for all tubes.

Results with H-37RV. Using the strain H-37RV, determinations were made simultaneously on the effect of streptomycin alone, PAS alone, and the two drugs in combination in various concentrations. Eight series were made with double dilutions of PAS ranging from 10 µg/cc in Series 1 to 0.07 µg/cc in Series 8. Series 9 contained no PAS. In each series tubes labeled A through F contained double dilutions of streptomycin ranging from 0.74 µg/cc to 0.023 µg/cc, and tubes labeled G contained no streptomycin. Thus, Series 9 was actually a streptomycin assay; the G tubes in Series 1 to 8 were a PAS assay, and the G tubes in Series 9 were controls since they contained neither drug. Each dilution of drug or combination of drugs was made in triplicate, and turbidimetric readings were averaged.

Streptomycin alone caused complete inhibition of growth of H-37RV after 19 days in concentration of 0.74 µg/cc and almost complete inhibition with 0.37 µg/cc (Fig. 1).

PAS alone inhibited growth almost com-

---

[10] Vennesland, K., Ebert, R. H., and Bloch, R. G., *Science*, 1947, **106**, 476.

[11] Mimeographed communication from Department of Clinical Investigation, Parke Davis and Company, Oct. 21, 1947.

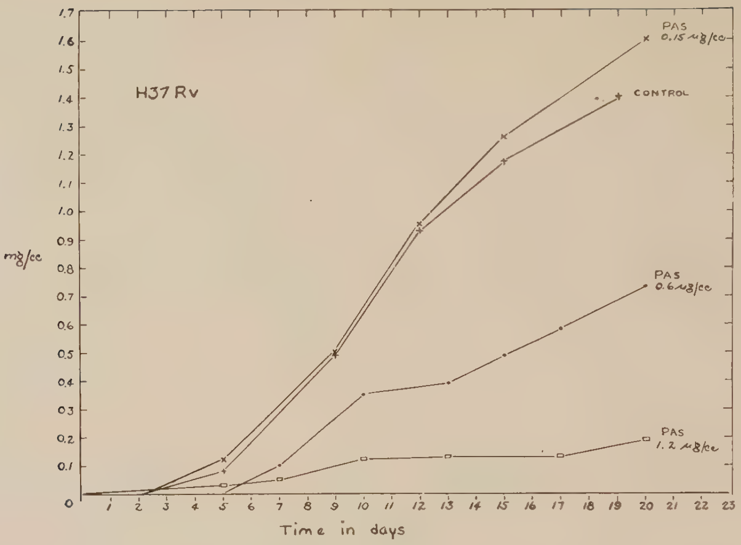


FIG. 2.
The effect of various concentrations of PAS on the growth of H-37RV.

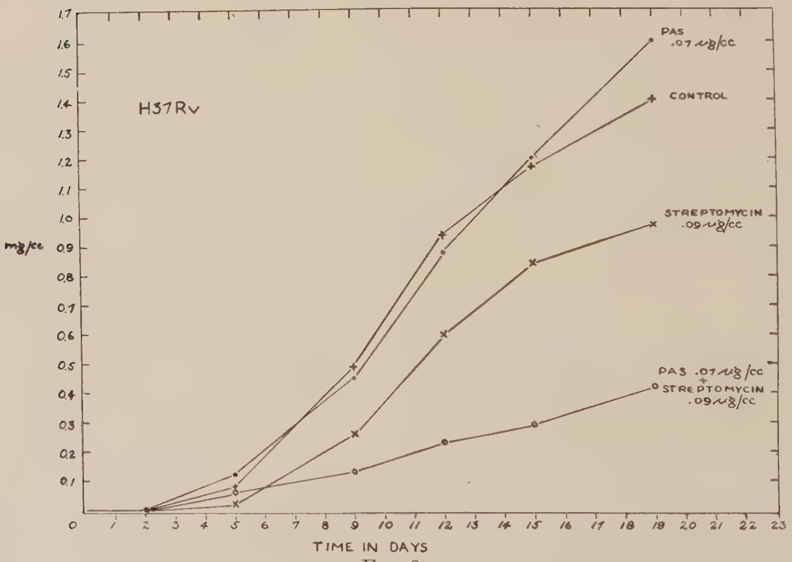


FIG. 3.
A comparison of the effect of various concentrations of PAS, streptomycin, and their combination on the growth of H-37RV.

pletely in concentration of 1.2 µg/cc and 0.6 µg of PAS per cc produced partial inhibition (Fig. 2). It was found in this and other experiments that PAS does not produce the sharp end-point of complete inhibition which is found in the case of streptomycin. Even concentrations of PAS as high as 10 µg/cc did not completely inhibit growth after 20 days of incubation.

The combination of streptomycin and PAS produced a greater inhibition of growth of H-37RV than either drug alone. The most favorable combination of drugs for demonstrating this synergistic effect was a concentration of one drug just below that necessary to produce fairly complete inhibition together with very small concentrations of the other drug. Thus 0.09 µg of streptomycin per cc

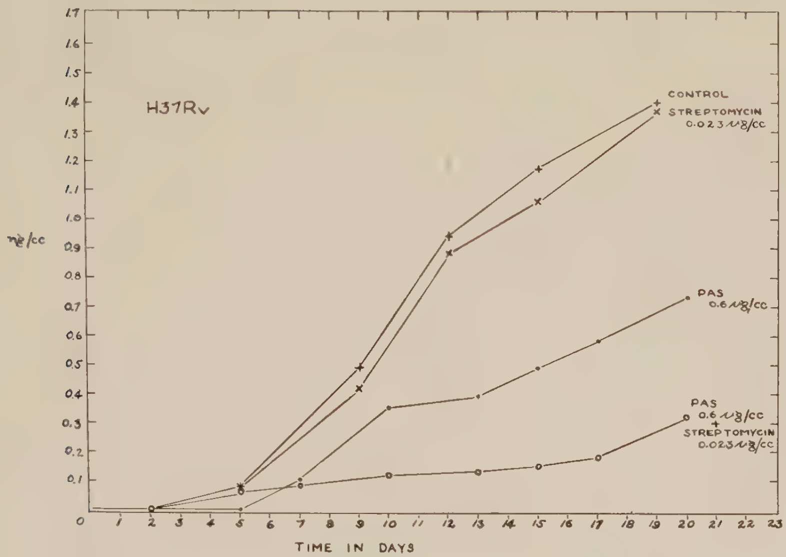


FIG. 4.

A comparison of the effect of various concentrations of PAS, streptomycin, and their combination on the growth of H-37RV.

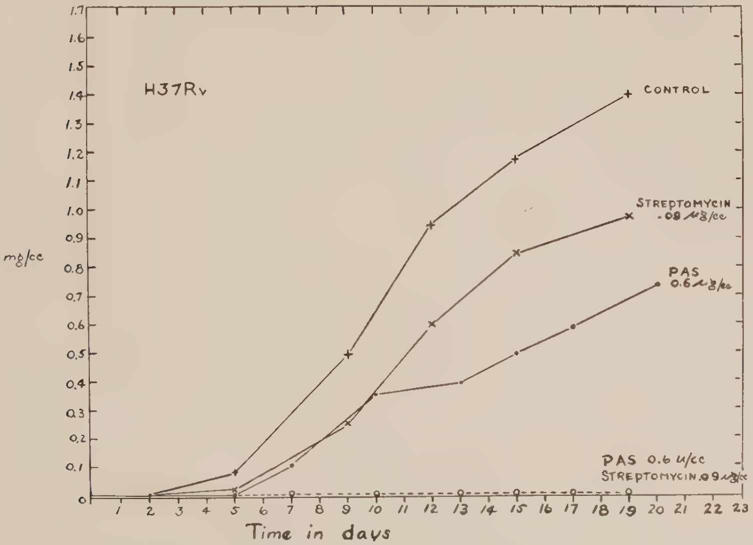


FIG. 5.

A comparison of the effect of various concentrations of PAS, streptomycin, and their combination on the growth of H-37RV.

produced only slight inhibition of growth, although 0.18 µg/cc produced fairly marked inhibition (Fig. 1). PAS alone in concentrations of 0.3, 0.15, and 0.07 µg/cc produced no inhibition. The combination of 0.09 µg of streptomycin per cc with each of these small concentrations of PAS, however, produced marked inhibition (Fig. 3).

Similarly, a concentration of 0.6 µg of PAS per cc produced only moderate inhibition of growth, and a concentration of 0.023 µg of streptomycin per cc produced none, but a combination of drugs in these concentrations caused increased inhibition (Fig. 4). The combination of 0.09 µg of streptomycin per cc and 0.6 µg of PAS per cc resulted in com-

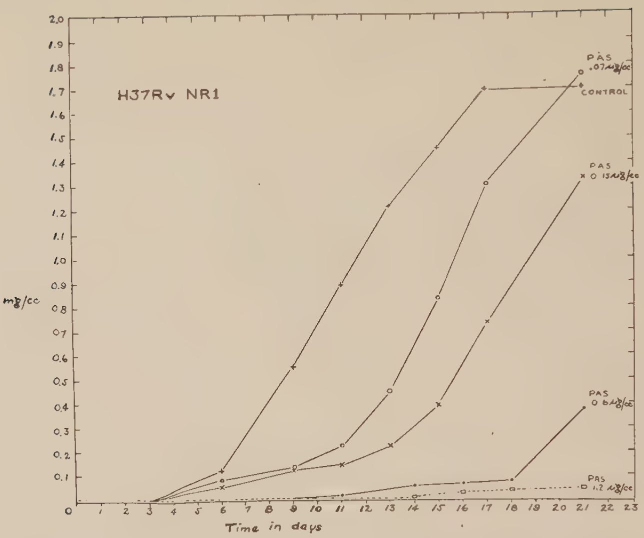


FIG. 6.

The effect of various concentrations of PAS on the growth of a streptomycin-resistant strain of virulent human tubercle bacilli (H-37RVNR1).

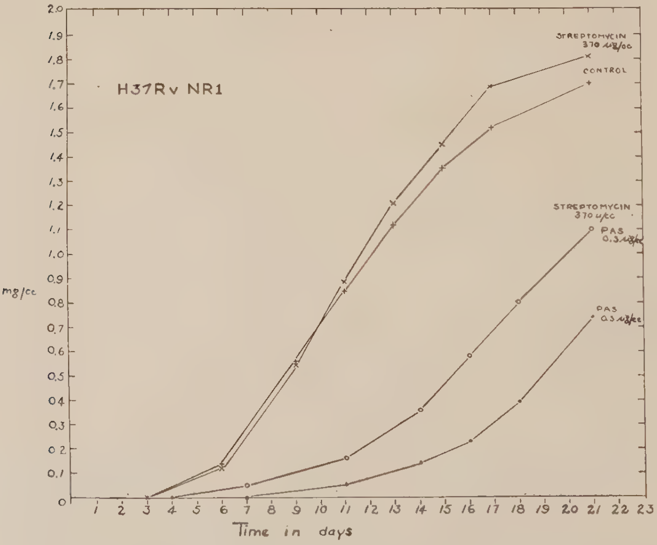


FIG. 7.

A comparison of the effect of 0.3 mg of PAS, of 370 mg of streptomycin, and of their combination on the growth of H-37RVNR1.

plete inhibition of growth (Fig. 5).

Results with Streptomycin-Resistant Strain. In this experiment using the streptomycin-resistant variant H-37RVNR1 the procedure was the same as that outlined above except that 10 × dilutions of streptomycin were made in Tubes A through F in all series in concentrations ranging from 3700 μg/cc to 0.037 μg/cc.

Streptomycin alone produced no inhibition of growth of this resistant strain even in the highest concentrations. It appeared that the streptomycin-resistant strain was slightly more sensitive to PAS alone than H-37RV, although the inhibiting concentrations were in the same general range (Fig. 6).

The combination of streptomycin and PAS caused no increase in the amount of inhibition on this resistant strain; in fact, there was slightly less inhibition with the combination of 370 μg of streptomycin per cc and 0.3 μg of PAS per cc than with PAS alone (Fig. 7).

Discussion. Relatively low concentrations of para-aminosalicylic acid (PAS) inhibit the growth of virulent human tubercle bacilli *in vitro*. It is worth noting that the concentration of drug necessary to inhibit growth varies considerably with the size of the inoculum. Thus more than 100 × the amount of drug is necessary to cause inhibition if an inoculum of 1.0 mg of tubercle bacilli per 10 cc of medium is used instead of 0.1 mg. This is not true of streptomycin. In experiments simultaneously carried out with both drugs concentrations of 1.48 μg of streptomycin per cc produced almost complete inhibition of growth using a large inoculum, whereas only slight inhibition was obtained with 100 μg of PAS per cc.

There is no question that the combination of streptomycin and PAS in critical concentrations is more effective than either drug alone if the organism is streptomycin-sensitive. These *in vitro* studies agree with the results of animal experiments, which will be reported elsewhere.

It appears that there is no increase in the inhibitory effect of the combination of streptomycin and PAS on the growth of a streptomycin-resistant strain if the streptomycin alone is ineffective (Fig. 7). If, however, a high concentration of streptomycin causes some inhibition, a combination of this amount of streptomycin with PAS will cause greater inhibition than either drug alone. Thus a streptomycin-resistant strain of virulent human tubercle bacilli, isolated from the lung of a patient on autopsy, was partially inhibited by 370 μg of streptomycin per cc. Using this strain the inhibitory effect of PAS was enhanced by the addition of 370 μg of streptomycin per cc.

Summary. 1. The *in vitro* growth of a virulent strain of human tubercle bacilli, H-37RV, is inhibited by 0.74 μg/cc of streptomycin. 2. The *in vitro* growth of H-37RV is inhibited almost completely by a concentration of 1.2 μg/cc of PAS. 3. A moderately inhibiting concentration of either streptomycin or of PAS when combined with a completely non-inhibiting concentration of the other drug produces a marked enhancement of inhibition of the *in vitro* growth of H-37RV. 4. *In vitro* growth of a resistant strain of human tubercle bacilli, H-37RVNR1 is inhibited by 1.2 μg/cc of PAS. 5. The inhibition of growth of this resistant strain is not enhanced by the addition of streptomycin.

We gratefully acknowledge the generosity of Parke Davis and Company, who supplied the para-aminosalicylic acid.

16447

Vitamin A and Thyroid Iodine after Reticuloendothelial Cell Block.*

B. Lionel Truscott and Dulal P. Sadhu.† (Introduced by W. U. Gardner.)
From the Department of Anatomy, Yale University School of Medicine.

The apparent antagonism between vitamin A and thyroid activity has stimulated investigations designed to clarify further the problem, and to determine the nature of this antagonism. It has been shown[1,2] that excess

---

* This research was aided by a grant from the Fluid Research Fund of Yale University.

† Special Fellow.

[1] Logaras, G., and Drummond, J. C., *Bioch. J.*, 1938, **32**, 964.

[2] Sadhu, D. P., and Brody, S., *Am. J. Physiol.*, 1947, **149**, 400.

TABLE I.

Series	Body wt (g)	Serum iodine γ/100 ml	Thyroid iodine γ/100 mg	Muscle iodine γ/g	*Vitamin A* Serum I.U.%	*Vitamin A* Liver I.U.%
Control (20 animals)	322	3.21 ± 0.12	90.4 ± 3.3	0.2243 ± 0.0586	85.0	408.0
Experimental (10 animals)	313	2.96 ± 0.48*	107.8 ± 4.8†	0.2485 ± 0.0252	53.2	411.2

* $p < .05$.
† $p < .01$.

vitamin A reduces thyroid size and lowers the hypermetabolism of animals given thyroxine. In studying the mechanism of this action, the authors[3] showed that hypervitaminosis A reduced the protein-bound iodine in the liver and thyroid, and increased the level of protein-bound iodine in the serum of rats. That the low level of hepatic iodine was not due to a general depression of body stores was confirmed by the elevated values obtained in skeletal muscles and in the pituitary gland.

The results described suggest the possibility that the rate of destruction of thyroxine by the liver is reduced, resulting in an increase in circulating thyroxine. It is known that any significant reduction of hepatic tissue, such as in subtotal hepatectomy, increases thyroxine activity[4] possibly by virtue of an elevated value of thyroxine in the blood. Furthermore, because excess vitamin A is stored in the Kupffer cells,[5-8] reducing their absorptive power,[9] it is conceivable that the vitamin impairs hepatic destruction of thyroxine by loading these cells. If this were the case, intraperitoneal injections of India Ink, by a similar mechanism, might furnish results like those obtained in hypervitaminosis A: increased circulating thyroxine and reduced thyroid size, with a decrease in thyroid iodine.

Adult male albino rats, placed on Purina Laboratory chow, were given intraperitoneal injections of 5-8 cc of a 5% suspension of India Ink every other day for 15 days. At the end of this time the livers were deeply stained, enlarged, and exhibited numerous points of adhesion to the peritoneal covering of the stomach and duodenum; microscopically there was widespread loading of the reticuloendothelial cells with the ink. The thyroid was not noticeably enlarged. Vitamin A was determined by the antimony trichloride method, using a Lumetron photoelectric colorimeter and protein-bound iodine was determined by the method of Salter and McKay.[‡][10] The results are shown in Table I. The iodine content of the thyroid was significantly increased to 107.8 γ % as contrasted with the control value of 90.4 γ %; it will be noted also that this is not accompanied by any appreciable change in thyroid size. Neither protein-bound serum nor muscle iodine were affected by the experimental conditions.

Serum levels of vitamin A were significantly lower than the normal although the hepatic stores of the vitamin were essentially unchanged. The lack of parallelism between serum and liver values, however, should not be unduly emphasized because low levels in the serum are known to exist in the presence of normal or increased stores of hepatic vitamin A.[8] The normal level of the latter offers further evidence that the vitamin accumulates appreciably in the reticuloendothelial cells of the liver only when excessive amounts are administered.

---

[3] Truscott, B. L., and Sadhu, D. P., *Anat. Rec.*, 1948, **100**, 87.

[4] Kellaway, P. E., Hoff, H. E., and Leblond, C. P., *Endocrinology*, 1945, **36**, 272.

[5] Hirt, A., and Wimmer, K., *Klin. Wchnschr.*, 1940, **19**, 123.

[6] Popper, H., and Greenberg, R., *Arch. Pathol.*, 1941, **32**, 11

[7] Popper, H., and Brenner, S., *J. Nutr.*, 1942, **23**, 431.

[8] Popper, H., and Steigmann, F., *Arch. Int. Med.*, 1943, **72**, 439.

[9] Huzita, Y., *Japan J. Obstet. Gynecol.*, 1938, **21**, 45.

[‡] The authors wish to thank Dr. William T. Salter for placing laboratory facilities at their disposal for the iodine determinations.

[10] Salter, W. T., and McKay, E. A., *Endocrinology*, 1944, **35**, 380.

The results described above are markedly different from those encountered in hypervitaminosis A. Blockade of the reticuloendothelial cells by India Ink is followed by an increase in protein-bound thyroid iodine, unaccompanied by changes in thyroid weight. It is obvious that if excessive amounts of vitamin A lessen hepatic destruction of thyroxine, this action cannot be due to an accumulation of the vitamin in the reticuloendothelial cells of the liver. That the effects must be explained by a different mechanism is further indicated by the lack of concomitant elevation in circulating thyroxine in the experiments discussed. The dissociation between the latter and protein-bound iodine in the thyroid is of interest because, in normal rats, the level of circulating thyroxine appears to be dependent upon the thyroxine content of the gland and is limited by the capacity of the gland to produce thyroxine.[11]

In view of the normal thyroid weight of our animals, it would seem probable that both production and storage of thyroxine is increased, although it is released in normal amounts into the circulation. Our studies on hypervitaminotic animals suggest that vitamin A may lessen hepatic destruction of thyroxine, with a consequent hyperthyroxinemia and increase in pituitary iodine; the result would be a depression of thyrotropic hormone secretion[12,13] and, eventually, a reduction in thyroid size. No explanations can be advanced as yet for the results described in the present paper. Further studies are necessary to determine the underlying mechanism producing these effects.

Summary. 1. Protein-bound iodine was decreased in the liver and increased in the thyroid after reticuloendothelial cell block; increased production and storage of thyroxine is indicated by the data presented. 2. Hepatic vitamin A was not affected by the experimental conditions, although serum vitamin A was decreased. 3. The bearing of these results on those of hypervitaminosis A is briefly considered.

---

[11] Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, 1946, **165**, 217.

[12] Adams, A. E., and Jensen, D., *Endocrinology*, 1944, **35**, 296.

[13] Sadhu, D. P., *Am. J. Physiol.*, 1948, in press.

16448

Ocular Refractive Changes Accompanying High Blood Sugar in Alloxan-Treated Dogs.

ARTHUR LAYTON AND J. M. D. OLMSTED.*

From the Division of Physiology, University of California, Berkeley, Calif.

Disturbances in the vision of diabetic patients are frequently observed which involve no apparent pathological changes in the eye. Since they may be remedied by a suitable correction lens, the phenomenon appears to be one of refractive change. These disturbances may be either in the direction of myopia or hypermetropia. In general, myopic changes are said to occur after the onset of diabetic conditions, but before the start of therapy; hypermetropic changes are said to occur after the start of therapy. Myopia may persist, but hypermetropia is always temporary, the average duration being 2 to 4 weeks.[1-3]

---

* Aided by a grant from the Research Board of the University of California.

[1] Granstrom, K. O., *Acta Ophth.*, 1933, **11**, 3.

[2] Duke-Elder, W. S., *Brit. J. Ophth.*, 1925, **9**, 167.

[3] Hudelo, A., *Arch. d'Ophth.*, 1930, **47**, 70.

The most acceptable theories to account for these disturbances in vision are all based on the assumption that the change occurs in the lens of the eye. It is supposedly brought about by changes in the osmotic pressure of body fluids, which in turn are the effect of variations in the blood sugar from the normal level.

For example, Granstrom[1] has suggested that salt retention by the tissues and loss of tissue fluid at the time of high blood sugar might increase the refractive index of the lens and result in myopia; with decreasing glycemia and glycosuria after the application of therapeutic measures, increased fluid uptake by the lens would result in a decrease in the refractive index, *i.e.*, hypermetropia. Duke-Elder,[2] on the other hand, suggests that with high blood sugar and salt retention, the blood is so diluted by body fluids that its osmotic pressure is lowered, and with it that of the aqueous humor, the result being that the lens swells and causes myopia; with a fall in blood sugar, the lens would shrink and cause hypermetropia. He cites cases of three human patients to uphold this theory; in one myopia varied directly as the blood sugar; in another, hypermetropia developed when there was a sudden decrease in blood sugar; in a third, myopia developed with an increasing blood sugar and persisted into coma and death. Therefore he concludes that the refractive power of the eye, or the myopia, tends to vary directly as the blood sugar level.

Procedure. To test under controlled conditions the hypothesis that changes in refraction accompany changes in blood sugar, 5 dogs were rendered diabetic and their state of refraction and blood sugars were followed as long as they remained alive. Observations were carried out during this period on 2 normal dogs as controls. It is to be noted that all the dogs were of undetermined age, neither very young nor very old, and in good health at the beginning of the experiment.

Fasting blood sugar values were determined according to the Schaffer-Hartman-Somogyi method using the macro-technic.

Refractive errors were determined by retinoscopy while the animals were under nembutal anesthesia and atropine cycloplegia. The refractive errors were denoted in the customary manner; positive values indicating the degree of hypermetropia and negative values, the degree of myopia. The visual axis had to be estimated in relation to the optic disc, but this was facilitated by the characteristic color of various portions of the dog's retina. Atropine (1% solution) was instilled into the conjunctival sac at 20-minute intervals, and retinoscopy was performed only at a minimum of 90 minutes after the initial installation, or 45 minutes after cessation of the pupillary response to bright light. Care was taken to keep the cornea moist with normal saline during the tests. Repeated observations have shown that retinoscopic findings on dogs under these conditions, like those on man, agree to within ± 0.25 D. Therefore it was assumed that changes of 0.25 D have no significance; changes of 0.50 have borderline significance; and changes of 0.75 D are definitely significant. The control dogs showed no significant changes during the 4 months they were kept under observation.

The 5 dogs were rendered diabetic by intravenous injection of alloxan (10% solution) in the proportion of 50 to 100 mg per kilo body weight. In some cases more than one injection was necessary to maintain a high

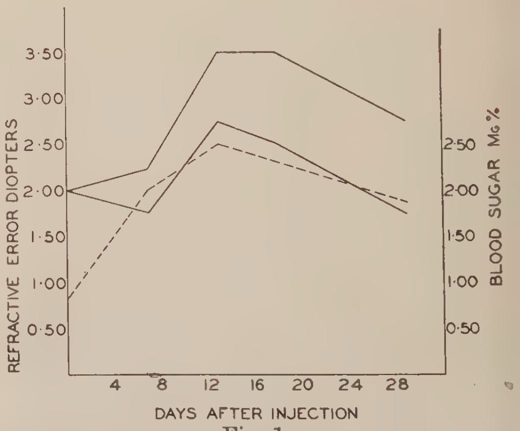


Fig. 1.

Results obtained on Dog No. 2 during the month following injection of alloxan. The broken line represents blood sugar. The unbroken lines represent the refractive error in the meridians of highest and lowest refractive power; therefore the vertical distance between these lines represents the degree of astigmatism.

blood sugar. The animals were fed twice daily a meal consisting of 200 g meat and 50 g sugar, which was more than ample for maintenance of weight. Water intake was unrestricted.

Results. The most striking of our results is shown in Fig. 1. The refractive change is in the direction of hypermetropia, not myopia, the change parallels the course of the blood sugar, and there are astigmatic changes as well. Such a definite refractive change, however, occurred in only 3 of the 5 dogs; the greatest refractive changes, in diopters, being in the 5 dogs, +1, +4, +.5, +1, and +.5, and the corresponding highest blood sugar values, in mg %, 340, 286, 440, 750, 500. No definitely significant change in two of the alloxan-treated dogs was observed, despite a very high level of blood sugar having been reached. No myopic changes whatever were observed in any of the 5 treated dogs.

In 1 dog an attempt was made to determine whether an alteration of the sympathetic-parasympathetic equilibrium would affect the refractive change. A sterile operation was performed in which the right superior sympathetic nerve was divided in the neck. In order to avoid the possibility of regeneration, the free ends of the nerve were sutured into the tissues some 3 inches apart. The only difference between the behavior of the 2 eyes of this dog after injection of alloxan was that the onset of the refractive change in the right eye appeared 2 days later than that in the left; the magnitude of the change was the same for both eyes.

Discussion. The significant observation in this set of experiments is that hypermetropic changes may occur at the time of acute or increasing hyperglycemia. This is in contradiction to the opinion of many investigators who state that in diabetic human patients hypermetropic changes occur only after the start of therapy.

It should be borne in mind that the alloxan-induced diabetes may differ from normally occurring diabetes or pancreatectomy diabetes. Thorogood and Zimmerman[4] state that coma is not common even in severe alloxan diabetes, and there is a lesser degree of acidosis and ketonuria. An increase in osmotic pressure within the lens might be the result of acidosis, in which state Krause[5] has suggested that certain proteolytic enzymes may act on the lens substance, producing fragmentation of protein with consequent increase of osmotic pressure. The entrance of water into the lens would represent the incipient cataractous state. Reports of transient diabetic cataract by Alt[6] and others suggest that myopic changes might be early cataractous changes which are reversible, and which do not progress to the point of opacification. Bellows and Chinn,[7] from their studies on swelling of the lens *in vitro,* conclude that there is a gradation of osmotic pressures ranging from external to internal layers, and water entering the lens tends to collect subcapsularly. Finally, there is evidence that myopic changes can be produced by lens swelling as in the transient myopia during sulfanilamide therapy. If myopia is the result of an acidosis, this factor may possibly be inoperative in alloxan diabetes.

Summary. Hypermetropic refractive changes may occur in the eyes of alloxan diabetic dogs at a time when the blood sugar level is rising or has reached a high level. Changes in the direction of myopia were not observed.

---

[4] Thorogood, E., and Zimmerman, B., *Endocrin.,* 1945, **37**, 191.

[5] Krause, A. C., *Biochemistry of the Eye,* Johns Hopkins Press, 1934.

[6] Alt, A., *Amer. J. Ophth.,* 1906, **23**, 294.

[7] Bellows, J. G., and Chinn, H., *Amer. J. Ophth.,* 1941, **24**, 979.

16449

Diet and the Duration of Thiopental Hypnosis in Rabbits.

BENJAMIN DE BOER. (Introduced by J. Auer.)

From the Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Mo.

Malnutrition[1] and hemorrhage[1,2] have been shown to increase the susceptibility to thiopental. Nedzel[3] noted the influence of diet upon the action of phenobarbital sodium, a long acting barbiturate, and indicated that rabbits fed a mixed diet were less affected by phenobarbital than those fed oats and water. This investigation was conducted to determine whether similar dietary factors affected the shorter acting drug thiopental, which appears to be detoxified by the liver.[4,5] The effect of nutrition upon the reactions of rabbits following a second injection of the same amount of thiopental a short time after apparent recovery from the first injection is also reported.

Materials and Methods. Thirty rabbits weighing 2.3 to 3.2 kg were given an intravenous injection of thiopental (pentothal sodium*). Each animal received 25 mg/kg of a freshly prepared 5% solution in 1 minute. Stage III anesthesia, indicated by depression of the corneal reflex, cessation of movements of the eyeball and muscular relaxation, ensued before the injection was completed. The hypnotic time was considered ended when the animal righted itself and drew its hind feet under the body into a normal sitting position. A second injection was given 1 hour after the first. A total of 76 paired injections are reported here.

Diets consisting of rabbit chow, oats and alfalfa, or oats were available with water at all times. At least 1 week elapsed after a change in diet before the hypnotic time was determined.

Results. The effect of diet upon the duration of hypnosis is indicated in Table I. All animals slept longer after the second injection than the first, indicating that some effects of the initial dose were present even after apparent recovery. Animals on diets of rabbit chow or oats and alfalfa showed an increase of 50% and 60% respectively in the duration of the second hypnotic interval. Rabbits upon a diet of oats for 1 week showed little difference in the initial sleeping time but the second hypnotic period was 90% longer than the first. The initial sleeping time increased about 65% after the animals were placed upon the oats diet for 4 weeks and the second sleeping period was more than two times that of the first. In many cases these animals were definitely depressed at the time of the second injection. Artificial respiration was often necessary immediately after the injection. In spite of this, 4 animals died. Artificial respiration was rarely necessary with animals on the other diets. Rabbits returned to a diet of chow after a 4-week interval on the oats diet showed a marked reduction in the hypnotic time within 2 weeks.

Animals on a diet of oats failed to gain weight comparable to those on the other diets, but appeared normal in other respects over a period of more than 3 months. When such animals were given chow or alfalfa, they rapidly increased in weight toward that of the control animals. The oats diet resulted in the excretion of an acid urine, pH of 6 or less as compared with pH of 9 with diet of rabbit chow and 7.5 to 8 with oats and alfalfa. When animals on oats diet were returned to a diet of chow the urine became

---

[1] De Boer, B., *Anesthesiology*, 1947, **8**, 375.

[2] Zweifach, B. W., Hershey, S. G., Rovenstine, E. A., Lee, R. E., and Chambers, R., *Surgery*, 1945, **18**, 48.

[3] Nedzel, A., J., *J. Lab. and Clin. Med.*, 1937, **22**, 1130.

[4] Shideman, F. E., Kelly, A. R., and Adams, B. J., *J. Pharm. and Exp. Therap.*, 1947, **91**, 331.

[5] Shideman, F. E., Kelly, A. R., Lee, L. E., Lowell, V. F., and Adams, B. J., *Fed. Proc.*, 1948, **7**, 255.

* The author is indebted to Abbott and Co., North Chicago, Ill., for a portion of the pentothal used in this work.

TABLE I.
Effect of Diet Upon Duration of Hypnosis Following 2 Intravenous Injections, 1 Hour Apart, of 25 mg/kg Thiopental.

Rabbit No.	Rabbit chow		Oats and alfalfa		Oats 1 wk		Oats 1 mo.		Oats 1 mo. chow 2 wk		Oats 1 mo. with $NaHCO_3$ 1 wk	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
1			15	33	26	45	42	82	22	31		
2			33	48	31	68	47	86	20	25		
3			24	34	25	62	33	72	14	25		
4			25	38	died							
5			25	38			58	135	21	35		
6			15	26			died					
7			22	34			48	246	33	47		
8			25	40			died					
9			12	29	25	48	51	98	40	59		
10			21	35	24	43	32	64				
11			20	34	25	42	died					
12			30	45	27	43	29	49	18	—		
13			30	42	26	50	51	107	16	39		
14	30*	42			26	49	43	55	19	31		
15	29	46			28	52						
16	25	39					36	101				
17	20	33					48	113				
18	24	34					32	68				
19	26	33					34	88	20	38		
20	28	41					41	—				
21	23	32					31	81	24	50		
22	27	44										
23	13	23										
24	27	45										
25	27	31										
26							48	103			39	60
27							35	64			24	35
28							43	57			19	32
29							33	101			40	59
30							32	66			26	42
Average	25	37	23	37	26	50	40	92	22	38	30	46

* Duration of sleep in minutes following intravenous injection.

alkaline within 1 week.

In a preliminary experiment to determine the relationship between acidity of urine and detoxification of thiopental 5 rabbits maintained upon a diet of oats were given sufficient $NaHCO_3$ orally to render the urine alkaline (pH 7.5 to 8.5). Before the addition of $NaHCO_3$ to their diet these animals showed an average sleeping time of 38 minutes after the first injection and 78 minutes after the second injection. One week later these animals with $NaHCO_3$ added to their diet showed a decrease in hypnosis to 30 minutes after the first injection and 46 after the second injection.

Discussion. That an injection of thiopental renders the animals more susceptible to subsequent injections has been reported in dogs.[1,6] The present experiments showed that a dietary condition resulting in greater depression after thiopental had the most pronounced effect upon a second injection. It is possible that rabbits on oats alone are in a state of malnutrition sufficient to account for their susceptibility. Preliminary work indicates that $NaHCO_3$ sufficient to cause the excretion of an alkaline urine in oats fed rabbits is beneficial in thiopental hypnosis.

Summary. The effect of 2 injections, 1 hour apart, of 25 mg/kg thiopental was studied in rabbits on 3 diets. Animals on a diet of oats slept much longer than those fed rabbit chow or oats and alfalfa. A diet of oats rendered the urine acid while the other diets were accompanied by excretion of an alkaline urine. $NaHCO_3$ fed orally to rabbits maintained on a diet of oats appeared to reduce the duration of hypnosis.

---

[6] Veal, J. R., and Reynolds, C., *South. M. J.*, 1938, **31**, 649.

16450 P

Role of the Liver in Tolerance to Morphine in the Rat.

George Fawaz. (Introduced by O. Krayer.)

From the Department of Biological Chemistry, American University of Beirut, Beirut, Lebanon.

Gross and Thompson[1] and Oberst[2] discovered that dogs and also man excrete morphine partly in a combined form. Since then, attempts have been made to study morphine metabolism in tolerant and nontolerant animals with a view of finding whether habituation was accompanied by a difference in metabolism.[1,3,4] Oberst[5] noticed that the glucuronic acid concentration of human urine increases with increasing dosages of morphine. Gross, Plant and Thompson[6] and Gross[4] found that liver damage causes an increase in the excretion of free morphine in both tolerant and nontolerant dogs, and that liver damage inhibits conjugation of morphine to a certain extent. Bernheim and Bernheim[7,8,9] discovered that liver slices conjugate morphine. Kidney and brain tissues were inactive.

It seemed of interest to investigate whether liver slices from tolerant rats are capable of binding more morphine than slices from normal rats.

Experimental Methods and Results. Essentially the technique of Bernheim and Bernheim[7] was followed except that double the amount of morphine was added to the slices. We have not used the silicomolybdic acid reagent because the depth of color was far from proportional to the concentration of morphine. We have utilized instead the Folin-Denis reagent according to Oberst.[10] Blank experiments were run which consisted of slices in Krebs-Henseleit buffer without added morphine. Morphine added to such suspension followed after 2 minutes by precipitation with trichloroacetic acid can be recovered quantitatively.

Rats were made tolerant by repeated daily injections of morphine hydrochloride, starting with 2 mg/100 g and reaching a dose of 20 mg/100 g in a period of 20 days. Preliminary results obtained with 9 normal rats and 4 tolerant rats showed a binding of 2.16 mg morphine per g wet slices per 3 hours for normal rats as compared with 1.86 mg for tolerant rats. With a different strain of rats the average binding by 4 normal rats was 1.39, as compared with 1.13 for 4 tolerant rats. The addition of glucuronic acid or more morphine to the suspension of slices resulted in no increase in the binding of morphine. The values given by Bernheim and Bernheim for normal rats in three different publications were: 2.31, 3.0, 3.5 mg morphine/g/3 hours.[7,8,9]

These preliminary results seemed to indicate that tolerant rats do not bind more morphine than normal rats. More direct experiments involving partially and completely hepatectomized rats were then undertaken. Tolerant rats were partially hepatectomized by removing under ether anesthesia the median and left lateral lobes which amount to 65% of the weight of the liver. These rats still tolerate the maximum dose of morphine (20 mg/100 g) injected half an hour after the operation and behave like normal rats that had been subjected to the same operation of partial hepatectomy and injected with saline.

---

[1] Gross and Thompson, *J. Pharm. and Exp. Therap.*, 1940, **68**, 413.

[2] Oberst, *J. Pharm. and Exp. Therap.*, 1940, **69**, 240.

[3] Thompson and Gross, *J. Pharm. and Exp. Therap.*, 1941, **72**, 138.

[4] Gross, Proc. Soc. Exp. Biol. and Med., 1942, **51**, 61.

[5] Oberst, *J. Pharm. and Exp. Therap.*, 1941, **73**, 401.

[6] Gross, Plant, and Thompson, *J. Pharm. and Exp. Therap.*, 1938, **63**, 13.

[7] Bernheim and Bernheim, *J. Pharm. and Exp. Therap.*, 1944, **81**, 374.

[8] Bernheim and Bernheim, *J. Pharm. and Exp. Therap.*, 1945, **83**, 85.

[9] Bernheim and Bernheim, *Arch. Biochem.*, 1946, **11**, 119.

[10] Oberst, *J. Lab. and Clin. Med.*, 1938, **24**, 318.

Tolerant rats were then subjected to "functional" hepatectomy by evisceration according to Russel.[11] The operation is performed under ether anesthesia and lasts about 10 minutes. Half an hour after the operation the rats walk around and appear quite "normal." These operated rats also tolerate the maximum dose of morphine and behave like normal operated rats injected with saline. That such asphyxiated livers are effectively cut off from circulation is shown by the fact that the blood sugar falls. However, the rate of fall of blood sugar varies greatly from one animal to the other. The survival time of such animals treated with saline injections varies from 3 to 20 hours. In accordance with the observation of Ingle and Sheppard[12] we observed no clear correlation between the time of survival and the level of blood glucose at death.

It is difficult to interpret these results in any other way than to mean that tolerance is not due to metabolism of the morphine in the liver, spleen or gastrointestinal tract.

---

[11] Russel, *Am. J. Physiol.*, 1942, **138**, 95.

[12] Ingle and Sheppard, *Endocrinology*, 1945, **37**, 377.

16451

Organ Antigen in the Early Chick Embryo.

A. Mandel Schechtman.

From the Department of Zoology, University of California, Los Angeles.

It was previously shown[1] that early embryonic stages of the chick contain one or more antigens occurring in the adult serum. In the present report evidence is presented for the presence in the early chick of a second type of antigenic substance(s) which is common to several adult organs and is serologically distinguishable from the antigens of blood and yolk.

Our previous work on the chick[1] indicated that the serum-antigen(s) in the early stages was exclusively of vitelloid nature, that is, serologically similar to certain constituents of the yolk. Non-vitelloid constituents of the serum appear in later stages of development. In attempting to demonstrate a new organ-antigen in the early embryo, it is therefore necessary to show that it can be distinguished from both blood and yolk antigens, since one group of antigens might be common to a given organ and blood, whereas another group might be common to blood and yolk.

Preparation of antisera. Brains were obtained from chicks near the time of hatching (19-20 days of incubation). The chicks were perfused with 0.85% NaCl solution injected into the left ventricle and allowed to flow from the cut jugular veins until clear of blood. The roof of the cranium was cut away, the brain removed to sterile Petri dishes and frozen over dry ice. When properly perfused the brain is of a chalky-white color. Accumulated brains were crushed while frozen and dried *in vacuo* at −10°C. The dried tissue, very porous and friable, was broken up with a sterile pestle and mortar and stored in tightly-stoppered bottles at −25°C.

The brain powder was stirred into ice-cold saline in proportion of 100 mg of dry powder for every 3 ml of saline. After mechanical stirring for about an hour at 0° to 3°C, the suspension was left overnight at 3° to 5°C, and then centrifuged at 3000 R.P.M.

---

[1] Schechtman, A. M., *J. Exp. Zool.*, 1947, **105**, 329.

TABLE I.
Ring (Precipitin) Tests With Anti-brain Sera Layered Under Extracts of Blood and Organs of the 19 to 20 Day Chick.

Anti-brain sera absorbed with extracts of	Test antigens (saline extracts)						
	Blood	Brain	Liver	Heart	Muscle	Yolk	Saline (Control)
Unabsorbed	+*	+	+	+	+	+	—
Blood	—	+	+	+	+	—	—
Brain	—	—	—	—	—	—	—
Liver	—	±†	—	±†	—	—	—
Heart	—	—	—	—	—	—	—
Muscle	—	±†	—	—	—	—	—
Unabsorbed	+	+	+	+	+	+	—
Normal serum§	—	—	—	—	—	—	—

* In 4 of 11 antisera the reactions with blood and yolk extracts were very weak.
† ± indicates that both negative and positive results were obtained. Explanation in text.
‡ Saline added in proportion to the greatest volume antigen used for absorption.
§ From uninjected rabbits.

for one hour during which the temperature never rose above 6°C. The slightly milky supernatant (brain extract) was stored at 3° to 5°C.

Twelve rabbits were injected intravenously with 0.5, 1.0, 2.0, 4.0, and 6.0 ml of the brain extract on alternate days. After an interval of 4 to 5 weeks a second series of injections was given, beginning with 0.5, 1.0, and 2.0 ml intraperitoneally, followed by the intravenous series already mentioned. Ten to 14 days after the last injection animals were bled from the heart.

Test antigens. Heart, muscle, liver, and the blood were obtained from the same chicks used to get brain, and were lyophilized and stored in the same manner as brain. The heart, and muscle from the upper leg, were cut out with scissors and washed briefly in saline before freezing. The liver was perfused while the lobes were gently moved about, until it was a butter-yellow color. The blood was obtained by cutting a window into the air space, pipetting 1 ml of 2.5% sodium citrate over the membrane covering the chorioallantois, and then puncturing several large blood vessels in the latter. Yolk was taken by pipette from the center of yolk of non-fertile eggs. The lyophilized test antigens were extracted in the manner described for brain.

Chick embryos from 3 developmental stages (primitive streak, early neural plate, and 4 to 5 somites) were used as test-antigens. The embryos were washed in 3 changes of saline, spread on glass slides, and the stage of development checked with a dissecting microscope. Excess fluid was drained off with a blotter, and the embryos were frozen and lyophilized. The dried tissue was scraped from the slides with steel needle-knives and extracted with saline added in the same proportions as for other test-antigens.

Precipitin tests. The test antigen extracts were too cloudy to be used in ring (interfacial) tests. Each antigen was therefore diluted to the point where a precipitation ring was readily seen. This dilution was considered the stock extract and from it dilutions of 1 to 8, 48, 144, 288 and 432 were prepared with saline. Approximately 0.08 ml of the test-antigen dilutions was layered over an equal volume of antiserum or normal serum in tubes of 3.5 mm inside diameter and 3 cm tall. The tubes were incubated at 37°C for ½ hour, stored at 18°C for 30 to 40 minutes, and read with fluorescent lighting against a black, opaque background.

Absorption of Antisera. Since the anti-brain sera cross-reacted with all the test-antigens, absorptions were carried out with each test-antigen separately, using the stock extracts. 0.5 ml of antiserum was mixed with 0.2 ml of each stock extract, and allowed to stand at room temperature for 15 minutes with frequent agitation. The precipitate was centrifuged down and the clear supernatant tested against the dilutions of the test-

TABLE II.
Ring (Precipitin) Tests with Anti-brain Sera Layered, Over Extracts of Early Embryos (Chick).

Anti-brain sera absorbed with extracts of	Test antigen (saline extracts)							
	Primitive streak	Early neurula	4 to 5 somites	Brain	Blood	Yolk	Egg-white	Saline (control)
Unabsorbed*	+	+	+	+	+	+	+	—
Blood	+	+	+	+	—	—	—	—
Brain	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	—	—	—
Heart	—	—	—	—	—	—	—	—
Normal serum†	—	—	—	—	—	—	—	—

* Unabsorbed antiserum diluted with saline in proportion to greatest volume of antigen used for absorption.

† From uninjected rabbits.

antigens. Absorption was repeated until ring tests were negative for all dilutions of the test-antigen used for absorption. The absorbed antisera were then tested against the test antigens. A plus sign in Tables I and II indicates a positive test in one or more dilutions of test-antigens.

Results. Table I shows that blood extract removes precipitins for yolk and egg-white as well as for blood. However, such blood-absorbed antisera retain precipitins for organ extracts. These results were obtained with all 11 antisera tested (one rabbit died during the injection series). This indicates that the brain contains one or more constituents antigenically distinct from those of blood, yolk, and egg-white. The antigen or hapten involved is not, however, organ-specific in the sense of being limited to brain alone. It should rather be referred to as a general organ antigen, since a majority of the anti-brain sera lost activity for all test-antigens after absorption with heterologous organs. Of the 6 antisera absorbed with brain, heart and muscle, 4 showed a complete loss of precipitins for all the test-antigens. In the 2 remaining antisera, one showed weak activity for heart and brain extracts after absorption with liver, while the other showed clear activity for brain after absorption with muscle extract.

The results given in Table II, obtained with 3 antisera, show that the early embryo contains one or more constituents antigenically distinct from those of blood and yolk. Since absorption of the anti-brain sera with brain, heart, or liver, removes precipitins for the embryonic tissue as well as for these organs, it must be concluded that the embryo contains the same general organ antigen (or combining groups) present in the differentiated organs. The early stages of the organism thus contain an antigen(s) similar to, or identical with, the vitelloid constituents of adult serum,[1] and another common to several and perhaps all differentiated organs.

Discussion. Whatever may be the specific source of the organ antigen, it seems, like the blood antigens, to be soluble in saline. This could not be deduced from the injection antigen which was a slightly turbid suspension. However, clear extracts were prepared from both adult organs and the early embryos by carefully pouring saline over the tissue so as to avoid dispersion of fine particles, and extracting in the cold without agitation for 48 to 72 hours. The clear supernatants gave positive ring tests with anti-brain serum absorbed with blood extract. Also in favor of the saline-solubility of the organ antigen is the fact that brain residue remaining after thorough extraction with saline, does not remove precipitins for any of the antigens tested. The specificity of the organ antigen as distinct from blood and yolk antigens, may be due to a lipoid (hapten) constituent since Heimann and Steinfeld[2] found that alcoholic extracts of brain can be used to produce antisera which are, in some instances, specific for both alcoholic and aqueous extracts of brain although cross-reactions with liver and heart occur with some antisera. Witebsky and Steinfeld[3] and Kopeloff and Kopeloff[4] found that anti-brain sera react with alcoholic extracts of brain.

---

[2] Heimann, F., and Steinfeld, J., *Z. f. Imm. forsch.*, 1928, **58**, 181.

Witebsky and Szepsenewol[5] found the Forssmann antigen in chicks at 24 hours incubation. Krichevsky[6] could not detect the antigen before the fourth day of incubation but he used aqueous suspensions whereas the above authors used alcoholic extracts. By the sixth incubation day, the Forssmann antigen can be detected in desanguinated chicks but not in their blood.[7] The antigen occurs in both blood and tissues of the adult.[8] The organ antigen involved in the present study is probably not the Forssmann antigen since the activity of the antisera for tissue extracts was not removed by thorough absorption with adult blood, which contains the Forssmann antigen.

Burke *et al.*[9] presented evidence for two brain antigens, one occurring in embryos from 160 hours to at least 312 hours but not in adult brain, and a second from 260 hours incubation to the adult. Unless we may assume that the antisera used were perhaps too weak to detect the antigen in the earlier stages, it must be concluded that these authors were working with substances different from the ones involved in the present study.

Summary. Substances of common antigenic character occurring in brain, heart, liver, and muscle can also be demonstrated in the primitive streak and neurula stages of the chick embryo. These substances are distinguishable from the antigens of blood and yolk by absorption and precipitin tests and are apparently soluble in saline.

---

[3] Witebsky, E., and Steinfeld, J., *Zentralbl. f. Bakteriol.*, 1927, **104**, 144.

[4] Kopeloff, L. M., and Kopeloff, N., *J. Immun.*, 1947, **57**, 229.

[5] Witebsky, E., and Szepsenwol, J., *C.R. Soc. Biol.*, 1934, **115**, 921.

[6] Krichevsky, I. L., *J. Infect. Dis.*, 1923, **32**, 192.

[7] Szepsenwol, J., and Witebsky, E., *C.R. Soc. Biol.*, 1934, **115**, 1019.

[8] Boyd, W. C., *Fundamentals of Immunology*, 1945, Interscience Publishers, Inc.,

[9] Burke, V., Sullivan, H. P., Petersen, H., and Weed, R., *J. Infect. Dis.*, 1944, **74**, 225.

16452

Infectivity of Murine SK Strain of Poliomyelitis Virus.*

EDWIN W. SCHULTZ AND S. COTTRELL WHITE.

From the Department of Bacteriology and Experimental Pathology, School of Medicine, Stanford University.

The history and characteristics of the murine SK strain have been described in several papers by Jungeblut and his associates.[1–5] One outstanding characteristic of the strain is its unusually high infectivity for mice, brain-cord virus suspensions rarely showing an ID_{50} titer under 10^{-7} by the intracranial route. This is in sharp contrast with the titers shown by the Lansing and other mouse adapted strains, but which, unlike the murine SK strain, still are paralytic for monkeys. In addition to its high infectivity by the intracranial route, Jungeblut found it infective

---

* Aided by the Howard Frost Poliomyelitis Research Fund.

[1] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

[2] Jungeblut, C. W., and Sanders, M., *J.A.M.A.*, 1941, **116**, 2136.

[3] Jungeblut, C. W., Sanders, M., and Feiner, Rose R., *J. Exp. Med.*, 1942, **75**, 611.

[4] Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, **75**, 631.

[5] Jungeblut, C. W., Feiner, Rose R., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 31.

by the intranasal, intraperitoneal, intravenous and subcutaneous routes, by "gavage" and by feeding bread soaked in virus suspension.[1,3,4] Old mice proved less susceptible than young mice and virus cultivated in tissue explants less infectious than brain-cord passage virus suspensions.[4]

It was of interest to us to extend these observations and to attempt to determine the tissue relationships of the strain following given types of exposure. Its high ID_{50} suggested that its relationships might differ from those of low titer strains, in portal demands and tissue cell requirements for growth. Moreover, the extent of damage that a given strain may inflict on the nervous tissue of a given host appears to be related to a considerable degree to the number of virus particles it is able to generate within that particular host.[6] This present strain, for example, which is high titer for mice, induces in this animal a highly acute encephalomyelitic type of infection. In guinea pigs, however, in which it becomes a low titer virus, it induces a characteristic poliomyelitic infection without noteworthy cerebral involvement.[2,5,7] In other words, its inability to reproduce itself as freely in guinea pigs as in mice becomes associated with a distinguishable difference in CNS involvement. Such quantitative differences in virus production growing out of individual host factors suggest that there may be little relationship between the ID_{50} of passage strains for individual kinds of laboratory animals and the amount of virus generated in terms of the natural host—man. Although the murine SK strain has failed to show either immunological or pathogenic relationships to the initial monkey passage SK strain,[7] thus leaving its true identity uncertain, it was of interest to make the following experimental observations on it.

Part of these studies relate to its transmissibility to animals other than mice. Those carried out with mice include experiments in which the animals were variously exposed to the virus, observations on the possible portals of entry in certain types of exposure, on possible sites of extraneural propagation of the virus, and on the distribution and character of the lesions induced. Virus assays and histological examinations were carried out on a number of animals. Those examined histologically were embedded *in toto* and sectioned transversely. More specifically, after removal of the skin and extremities, the body of each animal was placed in fixing solution, decalcified, cut into 3 parts (head, thorax and abdomen) and then processed for paraffin sections. Representative sections were stained by gallocyanin, Lentz A or the Giemsa method.

Most of the experiments relate to relatively natural modes of exposure, such as exposure to virus-contaminated food and drinking water, contaminated mouse jars, etc., and a few only to inoculations. Preliminary chemical blockade of the olfactory mucosa with zinc sulphate solution was employed in certain of the experiments. In some, virus grown in fertile eggs[8] was employed in place of brain-cord virus suspensions. Mice of known ages were employed throughout.

Infectivity by the intranasal route in mice of different ages. Six experiments were carried out in which the virus was instilled intranasally into mice of different ages. Five drops of a virus suspension were placed in slow succession on each nares of the etherized animal. In 2 experiments, the animals were instilled with a 10% mouse brain-cord virus suspension; in 4, with egg passage virus.[8] The results are shown in Table I.

These results confirm the observation by Jungeblut that young mice can be easily infected with this virus by the intranasal route. This holds also for the strain after it has been cultivated in eggs. Adult mice, however, showed themselves considerably more resistant to the egg passage virus, and more so to the latter than to the mouse passage

---

[6] Schultz, E. W., and Gebhardt, L. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 577.

[7] Schultz, E. W., and White, S. C., unpublished observations.

[8] Schultz, E. W., and Enright, J. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 8.

TABLE I.
Incidence of Infection in Mice Instilled Intranasally with Mouse Brain-cord and Egg Passage Virus Suspensions.

Exp.	Virus suspension	ID_{50} of virus susp.	No. of mice	Age, wks	% infection	Avg incubation period	Range of incubation period, days
1	Brain-cord virus	10^{-7}	10	3	100	4.0	3-5
2	” ”	10^{-7}	10	8	80	6.5	5-8
3	12th egg passage	10^{-5}	10	3	90	8.0	6-16
4	13th ” ”	10^{-5}	32	3	90	6.0	4-19
5	24th ” ”	10^{-6}	10	3	90	6.0	4-11
6	25th ” ”	10^{-6}	10	10	20	8.0	8

virus (cf. experiments 2 and 6).

Sections were made of 11 paralyzed mice inoculated with brain-cord virus suspensions by the intranasal route, and of 4 inoculated with the 13th egg passage virus by this route. Lesions were observed in the olfactory bulbs in only 2 of the first group and in only 1 of the second. These consisted of areas of necrotic mitral cells in one or both bulbs, with a sparse scattering of PMN cells. Those which showed lesions in the bulbs also showed involvement of the brain. On the other hand, brain lesions were observed in 3 animals which showed no lesions in the bulbs. Cord lesions were observed in all but 2 of the series. These animals had been infected with egg passage virus. Both had shown paralysis before they were sacrificed for histological examination. It should be added, however, that the lesions produced by this strain tend to be very acute, often with little cellular reaction, and that neurocytological changes in the absence of definite neurophagocytic or inflammatory reactions were not counted as positive observations. No changes were observed in the visceral or extranervous tissues.

Effect of chemical blockade on infectivity of the virus by the intranasal route. Seven experiments were carried out to determine whether infection by the intranasal route could be prevented by earlier intranasal instillations of a 1% solution of zinc sulphate.[9] In the first experiment, this solution was instilled only once by slowly placing 5 drops of the solution on each nostril of the anesthetized animal, in the second, the instillation was repeated at the end of 5 days, and in the remaining 5 experiments, the nasal passages were forcibly irrigated with the aid of a small pipette fit snugly into the nasal orifices. All of the subsequent virus instillations consisted of 10% virus tissue suspensions, either of mouse brain-cord material or of egg passage virus material. The results are given in Table II.

It will be noted that there was little difference in the incidence of infection between the zinc sulphate-treated groups and the controls. The more noticeable difference was observed in the 2 groups challenged with egg passage virus suspensions which had a considerably lower virus content (ID_{50} at 10^{-5}) than the brain-cord virus suspension (10^{-7}) employed, but even in these 2 groups the difference is not great enough to suggest that the virus depends upon the integrity of olfactory mucosa for its passage to the CNS.

Sections were prepared from 5 of the infected zinc sulphate-treated mice. All but 1 showed that the olfactory mucosa had been fully contacted by the zinc sulphate solution. This was evidenced by the extensive replacement of the normally thick and characteristic epithelium by one less thick, with cells in abnormal arrangement, and which could be easily recognized as a mucosa of the "reparative type." No involvement of the olfactory bulbs was observed in 4 of these animals. One animal showed mitral cell necrosis in a limited area in one of the bulbs. This happened to be in the one animal which showed much less extensive damage of the olfactory mucosa. All 5 animals showed lesions in the brain, but definite cord lesions, associated with inflammatory cells were

---

[9] Schultz, E. W., and Gebhardt, L. P., *J. Infect. Dis.*, 1942, **70**, 7.

TABLE II.

Incidence of Infection in Groups of Intranasally Inoculated Mice Previously Treated Intranasally with Zinc Sulphate Solution, Compared with the Incidence in Untreated Control Groups.

		Control group				Zinc sulphate-treated group				
Exp.	Virus suspen.	No. of mice	% infection	Avg incubation period, days	Range of incubation period, days	No. of animals	No. of days $ZnSO_4$ treatment preceded virus instillation	% infection	Avg incubation period, days	Range of incubation period, days
1	BV*	10	100	3.0	2-6	10	3	100	3.0	2-4
2	BV	10	100	3.8	3-6	10	6	100	5.7	5-8
3	BV	10	100	4.5	4-7	10	2	90	4.0	3-6
4	BV	10	80	6.0	4-10	10	5	90	7.5	4-12
5	BV	10	90	5.0	4-6	10	10	80	7.0	5-9
6	EP14†	10	100	5.0	4-6	7	7	71	6.0	4-7
7	EP26	10	100	5.5	4-7	10	5	80	7.0	5-8

* BV = Mouse brain-cord virus suspension.
† EP = Egg passage virus, 14th (and 26th) passage.

TABLE III.

Results of Virus Assays on Olfactory Bulbs and Brains of Mice Instilled with Virus Intranasally. To Reduce the Tabulation, Only the End Results Obtained with the Individual Dilutions of the Tissues Are Given Here.

			End of 24 hr				End of 48 hr				End of 72 hr			
			Bulbs		Brain		Bulbs		Brain		Bulbs		Brain	
Exp.	No. of mice instilled intranasally	Incidence of infection controls	Dilution	Infectivity	Dilution	Infectivity	Dilution	Infectivity	Dilution	Infectivity	Dilution	Infectivity	Dilution	Infectivity
1	20	4/5*									10^{-3}	1/3	10^{-3}	2/3
			10^{-1}	0/3*	10^{-1}	0/3	10^{-1}†	3/3	10^{-1}†	3/3	10^{-4}	1/3	10^{-4}	1/3
							10^{-2}	2/3	10^{-1}	0/3	10^{-6}	2/3	10^{-6}	3/3
2	20	2/5	10^{-1}	0/3	10^{-1}	0/3	10^{-3}	1/3	10^{-2}	0/3	10^{-7}	2/3	10^{-7}	1/3
			10^{-1}	0/5	10^{-1}	0/5	10^{-1}	3/5	10^{-2}	2/5	10^{-1}	5/5	10^{-6}	2/5
3	20	5/5	10^{-2}	0/5	10^{-2}	0/5	10^{-2}	0/5	10^{-3}	0/5	10^{-2}	0/5	10^{-7}	0/5

* Denominator = number of mice inoculated; numerator = number which became infected.
† Not carried to high dilutions.

observed in only 1 of the animals.

Time when virus becomes demonstrable in the olfactory bulbs and brain after it is instilled intranasally. Three experiments were carried out to determine how soon after intranasal instillation the virus makes its appearance in the olfactory bulbs and in the brain. In all, 3 groups of twenty 3 to 4 week old mice were instilled with a 10% mouse brain-cord virus suspension as previously described. Five animals were then taken from the particular groups at the end of 24, 48 and 72 hours respectively, sacrificed by exsanguination while under ether anesthesia, and the olfactory bulbs removed, pooled and titrated. The brains were removed last and made into separate pools. Five mice in each major group were held as controls. Ten percent suspensions of these pools were tested first. When these proved positive, further dilutions were tested. The results are given in Table III.

It will be noted that the bulbs and brains did not show the presence of virus at the end of 24 hours. Although it was demonstrable at the end of 48 hours, the results do not point definitely to an olfactory bulb to brain sequence. While in Experiment 2 the olfactory bulbs did show virus in 48 hours and the brain not, the results obtained in the other 2 experiments fail to support a bulb to brain type of spread. In other words, these 3 experiments failed to throw light on the question of whether the bulbs following intranasal instillation are the first to become involved or become involved as a secondary extension from the brain. An attempt was made to obtain the answer by histological examinations.

Twenty young mice were instilled intranasally with a 10% brain-cord virus suspension. Five of these were sacrificed for histological examination at the end of 24 hours, 5 at the end of 48, 5 at the end of 72 hours and 5 were held as controls. All of the latter developed paralytic infections, so that the entire group appears to have been adequately exposed. The sacrificed animals were embedded *in toto* and sectioned as described earlier. In none of the 15 animals were lesions observed in the olfactory bulbs, brain or cord, nor were any lesions observed elsewhere which could not be explained on other grounds. The fact that there were no lesions observed in the bulbs as late as 72 hours after inoculation might appear to constitute strong evidence against the olfactory route being in any real sense a preferred pathway following intranasal inoculation. On the other hand, the fact that virus had been readily demonstrable in pools of olfactory bulbs from other groups of mice similarly instilled with virus, and this within 48 hours after its intranasal instillation (Table III), suggests that it may exist in the bulbs, even in high concentrations, without evident lesions in the bulbs.

While the 5 controls in this particular group of animals were not included in these histological examinations, 11 infected mice similarly inoculated were examined at other times and the results on these are described in an earlier section of this paper. However, as a further means of determining whether involvement of the bulbs commonly results from a secondary spread of virus from the brain, sections were prepared from a small number of mice which had been infected by extranasal routes, including inoculations by the "clipped tail method," to be described in a later paper. Of 7 mice inoculated by such extranasal routes, 1 had been infected by the intracerebral route, 2 by the intraperitoneal route and 4 by the clipped tail method. All showed involvement of the brain. Four of these showed in addition involvement of the olfactory bulbs. One of these 4 had been inoculated by the intracerebral route, 1 by the intraperitoneal route and 2 by the clipped tail method. The most extensive involvement of the bulbs was observed in 1 of the latter animals, in which most of the mitral cells in both bulbs had become necrotic. From these results then it is evident that the virus spreads readily from the brain to the bulbs.

Infectivity of virus incorporated in the drinking water of mice. Thirteen experiments were carried out in which virus was incorporated in the drinking water of mice. A total of 210 mice was so exposed to virus. Part of the experiments were carried out with 1% virus suspensions, part with 10% suspensions, both made up in sterile distilled water. Two of the feedings with the 10% suspensions were carried out with mouse brain-cord virus suspensions and 4 with suspensions of egg passage virus. Each ml of the prepared water contained no less than 40,000 MID's, for mice. With one exception only (Experiment 4) 3 to 4 weeks old mice were employed. These were kept on the contaminated water from 1 to 5 days. They were placed on it in groups of 10 and these consumed about 100 ml of the water per day. It was supplied in bottles which delivered the water through a glass tube in drops which formed at the end of the tube and remained attached until removed by the animal. All of the animals were kept under observation for at least 2 weeks after the feeding period was ended. The results are given in Table IV.

The average incidence of infection in the 210 mice employed in these experiments was 47.5%. The average length of time the animals were fed virus was 3 days, and the average incubation period, counted from the first day of feeding, was 6.3 days. If we group the experiments in which mice 3 or 4 weeks of age were employed, and in which only a 1% brain-cord virus suspension was employed (Experiments 1, 2, 3, 5, 6), the incidence of infection then becomes 68.6% and the average incubation period 5.9 days. With 10% brain-cord virus suspensions (Experiments 7 and 8), the incidence of infection rose to 87%. A marked decrease in the incidence was observed in 8 weeks old mice (Experiment 4), in which only 1 out of 10 developed infection. Ten percent suspensions of egg passage virus proved less infectious than 1% suspensions of mouse brain-cord virus, the average incidence of infection in 90 mice so fed being 18%. Only 20% infection was realized on feeding the 26th egg passage virus over a period of 5 days. These results therefore suggest that the infectivity of the virus for mice was definitely decreased by its passages in eggs.

TABLE IV.

Incidence of Infection in Mice Given Mouse Brain-cord Virus, or Egg Passage Virus, in Drinking Water.

Exp.	No. of mice	Age, wks	Virus suspension	Virus tissue suspen., in %	No. of days animals were fed virus	% infection	Avg. incubation period, counted from 1st day of feeding	Range of incubation period counted from 1st day of feeding
1	10	3	BC*	1	5	80	5.5	4-6
2	10	4	BC	1	1	50	6.5	5-9
3	12	4	BC	1	2	50	8.0	6-14
4	10	8	BC	1	5	10	5.0	5
5	10	3	BC	1	5	90	4.5	3-8
6	30	3	BC	1	5	73	5.0	3-7
7	20	3	BC	10	2	75	6.0	4-8
8	20	3	BC	10	2	100	7.0	5-13
9	20	3	11th EP†	10	2	10	8.5	6-11
10	20	3	12th EP	10	2	30	6.0	2-12
11	20	3	14th EP	10	2	25	8.0	7-11
12	20	4	15th EP	10	1	5	11.0	11
13	10	3	26th EP	10	5	20	11.5	11-12

* BC = Mouse brain-cord virus suspension.
† EP = Egg passage virus suspension.

Sections were prepared from 9 mice infected by feeding the virus. All showed lesions in the brain and all but 2, well-defined lesions in the cord. Only 1 failed to show mitral cell necrosis in one or both olfactory bulbs. This could be regarded as evidence that the infection had taken place in most cases via the olfactory pathway. However, none of a number of mice removed from the feeding test within 1 to 2 days before symptoms should have developed showed lesions in the bulbs. This together with the observations mentioned earlier makes it seem probable that the bulbs in this series also had become involved by secondary extension from the brain. No lesions were observed in extranervous tissues of these mice.

Effect of zinc sulphate solution intranasally on the incidence of infection in mice later fed virus in drinking water. An experiment was carried out in which ten 3 weeks old mice were instilled intranasally with a 1% solution of zinc sulphate and 5 days later were placed on a 1% mouse brain-cord virus suspension in water for 5 days, along with 10 untreated mice as controls. Seventy percent of the treated group became infected as compared with 80% of the controls. This again suggests that the virus does not depend on the olfactory pathway for its access to the CNS.

Infectivity for newborn mice of mammary contamination. Two experiments were carried out in which a 10% mouse brain-cord virus suspension was applied to the mammary area of a nursing mother. In the first experiment this was applied to a mother of 8 newborns, 2 days of age, 3 applications of the virus being made on 1 day. All of the animals remained well up to the 5th day. On the morning of the 5th day, 1 newborn was sick, 1 was normal and 6 were missing, probably eaten by the mother. The remaining newborn succumbed on the 6th day and the mother herself developed paralysis soon after. In the second experiment, the virus was applied to a mother of 8 newborns, 4 days of age, with similar results. On the 3rd day, the partial remains of 1 newborn were found and 3 were missing. On the morning of the 4th day, 1 newborn showed paralysis. Three survived but the mother herself was found dead on the morning of the 9th day.

Infectivity of virus-contaminated mouse jars. Three experiments were carried out to determine how readily a contaminated environment serves to transmit infections. In the first experiment, the floor and lower portion of a clean battery jar was wet with a 10% brain-cord virus suspension. After the material had air dried, a small amount of sawdust was put into the jar, along with a supply of dry biscuits and drinking water, the latter in a bottle provided with a glass tube which delivered it as clinging drops. Five 3 weeks old mice were then placed into the jar. Four of these developed paralysis between the 6th and 8th day. In a repetition of this experiment, 6 out of 6 mice succumbed between the 5th and 12th days. In the second experiment, in which a jar was contaminated with a 1% suspension of infected brain-cord tissue, only 1 out of 6 mice developed infection. A repetition of this experiment yielded 2 infections out of 6 mice employed. In the third experiment, a jar was contaminated with a 0.1% suspension of infected brain-cord tissue. In this none of the animals developed infection during the 21 day period of observation—the period set for all of these experiments. This experiment was repeated with 10 mice. Again, none of the animals developed infection. These observations suggest therefore that while infections can be induced in this way, it requires a rather heavy contamination of the environment to bring this about. The possibility that latent infections may have been induced in some of these animals was not investigated.

Infectivity by the corneal and conjunctival routes. Ten mice were inoculated by placing one drop of a 10% brain-cord virus suspension into the conjunctival sac of one eye after a light scarification of the cornea. Seven became infected. However, of 20 mice similarly inoculated, but without scarification of the cornea, only 2 developed infection. Whether this low incidence was due to a reported "poliocidal" constituent in tears[10] or to some other factor was not determined.

Infectivity by the skin route. Two groups of 10 mice each were inoculated by applying a 10% suspension of brain virus to a small area of the skin on the abdomen and then introducing the virus into the skin by the multiple puncture method. Six in the 1st group and 7 in the 2nd group developed infections.

Relative infectivity by the intraperitoneal and intracranial routes. An experiment was carried out in which the infectivity of mouse brain-cord virus by the intraperitoneal route was compared with its infectivity by the intracranial route. Using a uniform dose of 0.03 ml and 6 mice for each of these 2 routes, its ID_{50} by the intracranial route was 10^{-7}, and 10^{-6} by the intraperitoneal route. With a dose of 0.1 ml by the latter route, in 6 mice, its ID_{50} remained at 10^{-6}.

Infectivity by the rectal route. Three groups of 10 mice each were inoculated by instilling 0.2 ml of a 10% mouse brain virus suspension into the rectum of each mouse with the aid of a Pasteur capillary pipette. The pipette employed was made with a well-rounded point to avoid injury to the tissues and the instillation was carried out in a way that would minimize trauma. Four to 6 weeks old animals were employed. Thirty percent became infected in each of the 3 groups, with an average incubation period of 6 days. In a preliminary experiment in which the instillations were less carefully carried out and some bleeding was induced, 8 out of 10 mice became infected.

Infectivity by the intravenous route. Ten 3 weeks old mice were inoculated by the tail vein, chiefly to determine the relative length of the incubation period following intravenous inoculation. All were injected with 0.1 ml of a 10^{-4} dilution of mouse brain-cord virus tissue. Nine of the group developed infection, with incubation periods ranging from 4 to 8 days and averaging 6.5 days.

Infectivity for animals other than mice. Shortly after reporting the adaptation of the original monkey passage SK strain to cotton rats and mice, Jungeblut and his associates reported the successful transmission of the mouse-adapted or "murine" strain to the guinea pig.[2,5,11] We have been able to confirm this observation and the fact that a typical poliomyelitic syndrome is induced in this animal; also the fact that the ID_{50} titer in mice of guinea pig passage material is much lower (10^{-3}) than that of mouse passage material (10^{-7}). Ten percent suspensions of both the murine and cavian passage virus proved infectious for hamsters by the intracranial route, but not for rabbits.

Four rhesus monkeys injected with large doses of a 10% suspension of the mouse passage virus by a combination of the intracranial and intraperitoneal routes failed to develop symptoms.

Brutsaert, Jungeblut and Knox[12] have recently reported attempts to adapt the MM strain to chicks. Soon after we had established that the murine SK strain can be cultivated in developing eggs,[8] several attempts were made by us to adapt egg passage virus, as well as mouse passage virus, to baby chicks. Chicks ranging from 2 days to 1 month of age were employed. These were inoculated with about 0.03 cc of a 10% suspension of either mouse brain or egg passage virus. Although suggestive clinical manifestations were induced in a few instances, we were unable to transmit the disturbance to new chicks, nor able to transmit any virus that might have been present in the brains of these chicks back to mice. Enright and Schultz[13] later observed that while murine SK virus can be readily propagated in fertile eggs containing embryos ranging from 5 to 14 days of age, embryos above 15 days of age no longer afford the pabulum necessary for growth of the virus. Attempts to gradually adapt the virus to older embryos proved unsuccessful.

---

[10] Jungeblut, C. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1534.

[11] Jungeblut, C. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 177.

[12] Brutsaert, P., Jungeblut, C. W., and Knox, Alice J., *Pediat.*, 1946, **29**, 350.

[13] Enright, J. B., and Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **66**, 541.

Summary. Observations are reported on the infectivity of the murine SK strain for mice based on certain types of exposure to the virus. Some of the infected mice were embedded *in toto,* sectioned, and examined histologically for evidence of virus multiplication in non-nervous as well as nervous tissue. Although infections occurred readily under various types of exposures to the virus, no lesions suggestive of virus activity were observed in any tissues or organs apart from the CNS. Infections by the intranasal route could not be prevented by prior intranasal instillations of zinc sulphate solution used to block the olfactory pathway as a portal of entry. Mice could be readily infected with virus-contaminated drinking water. Virus cultivated in developing eggs proved less infectious for mice when supplied to the animals in drinking water or food than mouse brain-cord virus suspensions. Old mice showed themselves less susceptible than young mice to certain types of exposure, but to other types of exposure the difference was less marked. Young mice could be readily infected by housing them in battery jars contaminated with virus, but it required a heavy contamination to bring this about. While a virus suspension dropped into the conjunctiva after light scarification of the cornea induced infection in 70% of mice, only 10% infection resulted when this procedure was not preceded by scarification of the cornea. Infection was induced in 60% of mice by applying the virus to the skin and then introducing it by the multiple puncture method. Infection was induced in 30% of mice on introducing the virus into the rectum. Young mice can be easily infected by the intraperitoneal route, this route being only slightly less sensitive than the intracranial route. The average incubation period following intravenous inoculation did not differ significantly from that observed following inoculations by other routes with similar amounts of virus. The virus induced a typical poliomyelitic syndrome in guinea pigs. In these animals it became a low titer virus. It proved infectious for hamsters but not for rabbits or monkeys. Attempts to adapt it to baby chicks were unsuccessful.

16453 P

Effect of Desoxypyridoxine on Reproduction in the Rat.*

MARJORIE M. NELSON AND HERBERT M. EVANS.

From the Institute of Experimental Biology, University of California, Berkeley, Calif.

It has recently been possible to show that an upset in the reproductive mechanism is quickly produced when normal adult female rats are shifted to purified diets deficient in pantothenic acid[1] or in pteroylglutamic acid.[2] The same purified diets in which the missing vitamins were present enabled normal reproduction to be evidenced. Such results induced inquiry as to the rapidity with which reproductive impairment might be evidenced by shifting similar healthy adult female rats to similar purified diets from which pyridoxine had been withdrawn. Preliminary studies, however, on pyridoxine deficiency started the day of breeding or one to two months prior to breeding showed only slightly adverse effects and, furthermore, the addition of succinylsulfathiazole to repress intestinal synthesis did not result in greater reproductive impairment. Recently, there has become available a very potent inhibitor of pyridoxine, "desoxypyridoxine" (2,4-dimethyl-3-hydroxy-5-hydroxy-methylpyridine) which is an antagonist to pyridoxine for the chick,[3] the rat[4,5] and the mouse.[6]

---

* Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: synthetic desoxypyridoxine, α-tocopherol, 2-methyl-1,4-naphthoquinone, and crystalline B vitamins from Merck and Company, Rahway, N. J.; synthetic d-biotin from Hoffman-La Roche, Inc., Nutley, N. J.; and synthetic pteroylglutamic acid from Lederle Laboratories, Inc., Pearl River, N. Y.

[1] Nelson, M. M., and Evans, H. M., *J. Nutrition,* 1946, **31**, 497.

[2] Nelson, M. M., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 289.

In the present communication we wish to report the reproductive behavior of normal adult female rats placed either on the day of breeding or shortly before it, on a pyridoxine-deficient diet to which desoxypyridoxine had been added.

Experimental. Normal female rats (Long-Evans strain), 3 months of age, were bred with normal males and placed on the pyridoxine-deficient diet† containing 0.5 mg % desoxypyridoxine on the day of breeding. Other groups of rats were maintained on this diet for 10, 15, or 20 days and then bred as rapidly as possible. Control rats were given the identical diet supplemented with 0.5 mg % pyridoxine on the day of breeding after

TABLE I.
Reproduction of Rats Maintained on Purified Diets Containing Desoxypyridoxine (0.5 mg %).

Day exp. diets started	No. of rats bred	Failed implantations %	Resorptions of implantations %	Litters of implantations %	Total No. young	Young born dead %	Avg size of litter	Avg wt of young, g
Pyridoxine supplemented: Day of breeding*	10	0	0	100	93	0	9.3 (6-16)	5.6
Pyridoxine deficient: Day of breeding	21	0	10	90	143	3	7.5 (3-11)	5.0
12 days before breeding	11	0	36	64	34	0	5.7 (2-9)	5.2
16 " " "	12	0	75	25	19	21	6.3 (5-8)	4.6
22 " " "	7	29	100	0				

* These rats were given the pyridoxine-deficient diet containing 0.5 mg % desoxypyridoxine 14 days (11-22) previous to breeding.

---

[3] Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 125.

[4] Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, 1947, **167**, 573.

[5] Emerson, G. A., *Fed. Proc.*, 1947, **6**, 406.

[6] Stoerk, H. C., *Fed. Proc.*, 1948, **7**, 281.

† The pyridoxine-deficient diet containing the antagonist was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco), 4% salts No. 4, and 0.5 mg % desoxypyridoxine. Crystalline vitamins per kg diet were: 300 μg d-biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5.5 mg synthetic pteroylglutamic acid, 5 mg thiamine HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid or amide, 50 mg calcium pantothenate, 400 mg inositol, and 1.0 g choline chloride. 5 mg pyridoxine HCl per kilo (0.5 mg %) was added to the diet for the control group. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg synthetic α-tocopherol, and 650 mg corn oil (Mazola).

being maintained on the deficient diet containing the antagonist for 10 to 20 days. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals.

Results. Table I shows that placing adult rats the day of breeding on the pyridoxine-deficient diet containing the antagonist resulted in a slight interference with reproduction, evidenced principally by the occurrence of resorptions in 10% of the group. When the rats were placed on this diet 10 to 20 days prior to breeding marked upsets in reproduction were observed. The percentage of resorptions increased directly with extension of the deficiency period, *i.e.* 36% of the rats resorbed after 12 days (11-13) of deficiency prior to breeding, 75% for those averaging 16 days (14-22) deficiency prior to breeding, and 100% of the rats showing the implantation sign, resorbed when the deficiency was extended to 22 days (18-27) before breeding. In the last group it may be noted that a failure of implantation also occurred to a significant extent (29%). The percentage of young born dead was markedly increased (*i.e.* 21%) in the group with 16 days deficiency prior to breeding and a significant decrease in the average weight of the young at birth was also observed. The tendency toward a decreased number of young per litter and decreased weight of the young at birth may be noted in all deficient groups. The normal reproductive behavior of rats supplemented with pyridoxine the day of breeding after 14 days (11-22) of deficiency previous to breeding, showed that the vitamin counteracted all the deleterious effects of the antagonist.

This study adds another dietary condition to those previously found to cause resorption in the adult female rat.[1,2] The rapidity with which the reproductive mechanism is disturbed in this experiment is similar to that previously found in the case of pantothenic acid deficiency. To be noted is the early appearance of the erythrocyte sign frequently observed in this study—on the 9th, 10th, and 11th days of gestation instead of the normal 13th, 14th and 15th days. In the rats not bred, there was an early suppression of the oestrous cycle despite the maintenance of body weight and an early appearance of the characteristic acrodynia on the paws (in 15 days for many rats), indicating the acuteness of the deficiency produced by the antagonist and the importance of pyridoxine for these functions.

Summary. The addition of the pyridoxine antagonist, desoxypyridoxine, to a pyridoxine-deficient diet resulted in marked reproductive upsets when normal adult female rats were placed on the diet only 10 to 20 days prior to breeding. Supplementation with pyridoxine on the day of breeding counteracted the adverse effects of the antagonist.

16454

Morphology of *Hemobartonella muris* as Revealed by Darkfield Microscopy.*

J. D. REESE AND EARL M. FOWLER. (Introduced by Douglas H. Sprunt.)

From the Department of Pathology and Bacteriology, University of Tennessee College of Medicine and the University of Mississippi School of Medicine.

During the course of studies on *Hemobartonella muris*, we have had occasion to observe the morphology of this organism with the aid of the darkfield microscope. Darkfield preparations of peripheral blood, made at the height of anemia in the rat, have been compared with blood smears made simultaneously and stained with one of the Romanowsky methods.

---

* Aided by a grant from the John and Mary R. Markle Foundation.

Weinman[1] has presented an excellent summary of the divergent points of view as to the morphology of this organism in the fresh state. These varying opinions are summarized in the following table, which is based upon Weinman's summary of observations from several sources.

I. Mayer, Borchardt, and Kikuth (darkfield and ordinary illumination): Dots only were found—these too few to correspond to the number of stained parasites.[2]

II. Ford and Eliot: Nothing suggestive by direct lighting either in hanging drop or unstained film—motionless parasites visible in darkfield.[3]

III. Dinger: Parasites present in darkfield—animated by Brownian movement.[4]

IV. Lauda: Parasites in darkfield are motionless.[5]

V. Bayon[6] and Perez[7]: Slow and sinuous motion of the organisms was observed in the red cells.

VI. Noe and Paez[8] and Rordorff[9]: Report rapid motion of the organisms.

VII. Levi[10]: Observed innumerable refractile granules, the majority free in the plasma, which showed 5 to 10 minutes lively motion, not of a Brownian type and then became quiescent.

In our own work, we feel that we have amply confirmed the variation in morphology reported for this organism as seen in the stained smear. The extremely fine granular forms reported by Vasiliu,[11] the dumbbell shaped organisms, the flame shaped diplococcoids, rods, arrangement in short chains, and indeed the entire range of morphological variation described by previous workers has been observed.

We felt that further use of darkfield examination of the blood of parasitized rats might be valuable. First, we believed that it might give us some conclusive evidence as to the position of the organism relative to the red cell; and second, that it might provide additional data as to motility of *H. muris*

Repeated examinations of peripheral blood of splenectomized animals with the darkfield microscope have been made, and, simultaneously, the same blood has been examined in the stained smear. Comparison of these two types of preparation has convinced us that there is, sometimes, a marked disparity between the number of organisms that can be seen in the stained preparation, and those seen by darkfield examination. Our studies have convinced us that *Hemobartonella muris* in the fresh preparation exhibits a motility which, though limited, is definitely in excess of Brownian movement. Use of darkfield preparations has further convinced us that this organism may be both intracellular and epicellular.

In reference to the first observation noted above, namely the disparity in number of organisms in the darkfield preparation and stained smear, some remarks are pertinent. We have noted that organisms seldom seem as numerous in the darkfield preparation as in one stained with Wright or Giemsa stains. When a darkfield preparation is made and immediately viewed, the most striking element is the granular form of the organism. These granular forms are observed in the cells and free in the plasma. Some difficulty is at first experienced in differentiating the organism from hemaconia. A little practice, however, enables one to make this differentiation without much difficulty. The hemaconia are more constant in morphology, they are larger and they are more refractile than is the granular variety of *H. muris*. These criteria allow one to differentiate the granular form of *H. muris* from hemaconia; differentiation, of course, of the longer form of *H. muris* from hemaconia presents no difficulty. Some practice in observation of darkfield preparations of fresh blood enables one to make out especially in the red cells, longer forms of *Hemobartonella muris* which are very thin.

---

[1] Weinman, David, *Transactions of the Am. Phil. Soc. N.S.*, 1944, **33**, 243.

[2] Mayer, M., Borchardt, W., and Kikuth, W., *Arch. f. Schiffs-u. Tropen-Hyg.*, 1927, **31**, 295.

[3] Ford, W. W., and Eliot, C. P., *J. Exp. Med.*, 1928, **48**, 475.

[4] Dinger, J. E., *Nederl. tijdschr. v. geneesk.*, 1929, **73**, 1910.

[5] Lauda, E., *Patho. Mikroorg.*, 3rd ed., 1930, **8**, 1073.

[6] Bayon, H. P., *J. Trop. Med.*, 1928, **31**, 29.

[7] Perez Aro, A., *Vida nueva*, 1933, **32**, 51.

[8] Noe, J., and Paez, R., *Rev. Inst. Bact. de Chile*, 1930, **1**, 20.

[9] Rordorff, R., *Gior. de bateriol. e immunol.*, 1933, **11**, 351.

[10] Levi, M., *Haematologica*, 1931, **12**, 1.

[11] Vasiliu, N., *Compt. Rend. Soc. de Biol.*, 1929, **102**, 173.

We feel it entirely likely that all of the morphological variations which are present in the stained smear are present in the fresh preparation. Because of lack of color contrast and the extremely delicate character of the organisms, these morphological variants are difficult to observe in fresh preparation. In the larger red cells particularly, we have observed a marked variation in the shape of the organisms found in relation to a single red blood cell. In the stained preparation, not only is the eye aided by the color contrast but the organisms, as might be expected, appear to be considerably larger than in the unstained preparation.

Motility is most marked when the darkfield preparation is first observed; it abates as time progresses. Motility of the forms free in the plasma, which is very marked when a preparation is first viewed with the darkfield microscope, diminishes as the fibrin network is formed. After the formation of fibrin, active motility is seldom observed. Whether this is due entirely to the fibrin formation or due simply to the age of the preparation, we are unable to say at the present time. The motility exhibited in the plasma is, we feel, unquestionably directional in character. Organisms traverse the field from one portion to another past intervening cellular structures in a rather slow but unidirectional movement.

Of considerable interest is the activity of those organisms which are related to the red cells themselves. We have observed single parasitized red cells over a relatively long period of time and have noted that those organisms which appear to be intracellular do not hold one fixed position but, on the contrary, may move about quite freely. At times, such parasites will be distributed over the extent of the entire cell; at times, they will all be arranged about the cell periphery. Organisms so related to the cell are often in constant motion. The motility of elongate forms is less likely to be vigorous than is the case of the granular ones. In those organisms which show considerable length, the slow sinuous motion reported by Bayons and by Perez has been repeatedly observed. We have also seen a bending of the elongated organisms in their central portion followed by extension to their full length. This alternate bending and extension appears to play some role in the propulsion of the organism across the field.

We have observed parasitized cells for prolonged periods without observing an organism to leave the confines of the cell. It is difficult for us to understand how such forms could be simply epierythrocytic as has been reported to be true in the case of *Bartonella bacilliformis*.[1] We have frequently seen forms which obviously occupied a position on the exterior of the cell, and such forms have been seen to separate from the cell and again to become free in the plasma. We are in agreement with McCluskie and Niven[12] that *H. muris* is found both on and in the red cell of the rat.

Summary. Darkfield examination of the blood of rats parasitized with *H. muris* shows that organism to have a motility which, though limited, is directional in character. Motility of the organisms in the plasma is diminished rapidly as the preparation ages. The forms seen in darkfield preparation are not limited to the granular variety; numerous variations in morphology are present and probably the entire morphological range as can be seen in the stained film. Our observations lead us to conclude that the organisms are both intracellular and epicellular in position in relation to the red cell, as well as being free in the plasma.

---

[12] McCluskie, J. A. W., and Niven, J. S. F., *J. Path. and Bact.*, 1934, **39**, 185.

16455

VI. Electrocardiographic Study of Heart and Effect of Vagotomy in Phosgene Poisoning.*

H. D. Bruner, R. D. Boche, M. H. Gibbon, and M. D. McCarthy.
(Introduced by I. S. Ravden.)
From the Harrison Department of Surgical Research, Schools of Medicine, University of Pennsylvania.

Bradycardia, salivation, vomiting, urination, and defecation regularly appear during or shortly after exposure to phosgene.[1–6] The bradycardia persists up to the critical anoxic phase and is clearly of neurogenic origin since vagotomy or atropine prevent or abolish it. These very early signs suggest an over-all, abnormally high parasympathetic tone which, therefore, was investigated by electrocardiography in order to: (1) confirm this clinical impression and assess the importance of parasympathetic tone in the etiology of the lung damage and (2) evaluate this sign of poisoning in relation to prognosis. The electrocardiograph used in conjunction with the electro-encephalograph also offered a means of detecting whether the central nervous system or heart was more affected by anoxemia in the critical and terminal stages.

Methods[†]. Thirteen healthy dogs were studied electrocardiographically using the 3 standard leads and the apical-left leg lead with the dog placed in the supine position on a shaped wooden board. This position according to preliminary experiments gave reproducible tracings. Lead II was used for following changes at short intervals. Samples of the other 3 were taken at longer intervals as indicated. Following several control tracings the dogs were exposed to phosgene in a dynamically operated chamber as previously described.[7] In the low concentration (0.5 mg per liter)—long duration (30 minutes) exposure, the leads were passed into the chamber and tracings were made throughout the gassing. In the equivalent high concentration (5.0 mg per liter)—short duration (3 minutes) exposure, tracings were started within 2 minutes after the end or 5 minutes from the beginning of gassing. Both exposures are an L(CT)99 for dogs.

The dogs on which electro-encephalographic records[‡] were also made had recovered fully from an operation at which metal guide plugs had been screwed in the temporal bones. Chlorided silver electrodes thrust through the overlying skin were used for recording.

Results. Excessive sinus arrhythmia was seen regularly as soon as 1½ minutes after beginning exposure to the low concentration. This was followed by sinus bradycardia which attained its maximum within the first half of the 30 minute exposure. The PR interval lengthened about 0.03 seconds during the middle of 10 minutes gassing and then shortened somewhat. Changes of the ST interval (or ST + T, because of difficulty in determining the onset of T) paralleled the

---

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

[1] Laqueur, E., and Magnus, R., *Z. f. d. ges. exp. Med.*, 1921, **13**, 31.

[2] Underhill, F. P., *The Lethal War Gases*, Yale Univ. Press, New Haven, 1920.

[3] Meek, W. J., and Eyster, J. A. E., *Am. J. Physiol.*, 1920, **51**, 303.

[4] Gibbon, M. H., Bruner, H. D., Boche, R. D., and Lockwood, J. S., To be published in *J. Thor. Surg.*

[5] Patt, H. M., Tobias, J. M., Swift, M. N., Postel, S., and Gerard, R. W., *Am. J. Physiol.*, 1946, **147**, 329.

[6] Rothlin, E., and Suter, E., *Helvetica. Physiol. et Pharm. Acta.*, 1946, **4**, 147.

[†] We wish to express our indebtedness to Doctor Samuel Bellet for his considerable help and continued interest in this project.

[‡] Carried out through the cooperation of Doctor Don Scott of the Johnson Foundation.

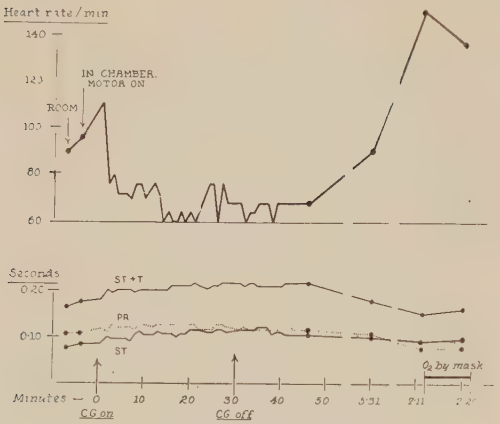


FIG. 1.

Changes in heart rate, PR, ST, and ST-T electrocardiographic waves (Lead II) during exposure to phosgene (0.52 mg per liter for 30 minutes, a L(CT)99 dose) and at subsequent representative intervals.

PR changes as shown in Fig. 1. Other electrocardiograph values remained essentially constant.

The first tracings taken in air after gassing at the high concentration for 3 minutes showed sinus bradycardia and lengthened PR and ST + T intervals, which attained their maximum during the next 5 to 10 minutes and then slowly regressed. The magnitude of change is of the same order as that in Fig. 1. The 2 types of exposure to phosgene evoked essentially identical cardiac responses regardless of the fact that the animals gassed by the high concentration technique were again breathing air when the maximal changes occurred.

In 2 dogs during the height of the bradycardia, ectopic areas assumed the pacemaker role—the ventricle in one case and probably the upper part of the bundle or the A-V node in the other. A third dog showed A-V block temporarily some 20 minutes after gassing began.

This combination of sinus arrhythmia, sinus bradycardia and prolonged conduction time may be considered pathognomonic or heightened vagal activity.

These findings regressed to normal as the heart rate increased during the second to sixth hour and showed no further change despite subsequent development of tachycardia coincident with development of pulmonary edema, anoxemia and a shock-like state of the circulation. Occasional transient inversions of the QRS and/or T waves in leads II and III occurred in the anoxic phase but nothing characteristic of cardiac anoxia.[7] Similarly in the electro-encephalographic tracings the waves characteristic of the dog continued despite severe cyanosis associated with stupor. The electro-encephalographic waves, however, died out shortly before the animal ceased to breathe whereas normal electrocardiographic complexes continued until after complete respiratory failure. The heart then showed bizarre ectopic and premature beats, various types of block and finally ceased. Terminal ventricular fibrillation was very brief or absent.

Oxygen therapy sufficient to abolish the cyanosis (Fig. 1) made no significant changes in the tracings and slowed the heart only slightly. Apparently the heart was not critically impaired even though the animal, as in Fig. 1, was soon to die.

No correlation could be discovered between changes of electrocardiographic measurements and either rate of development or severity of the syndrome. This confirms Underhill's[2] study of heart rate changes in relation to survival.

To investigate the possible role of excessive parasympathetic tone in the genesis of pulmonary damage, detailed histologic studies were carried out on the lungs of animals gassed before or after unilateral or bilateral cervical vagotomy done under local or evipal anesthesia. In other dogs the vagi were temporarily blocked during gassing by infiltration with procaine. In still other dogs atropine or physostigmine was administered to block or exaggerate, respectively, parasympathetic tone. Most of the dogs had been devocalized previously to eliminate complications from laryngeal paralysis.

The bradycardia was absent whenever the vagi were cut, or blocked by procaine, or the end organs blocked by atropine. Physostigmine appeared to have no special effects.

---

[7] Harris, A. S., and Randall, W. C., *Am. J. Physiol.*, 1944, **142**, 452.

All the other signs of parasympathetic response were present. Bradycardia developed as the procaine wore off *after* gassing was ended.

Interpretation of all these sections with reference to appropriate ungassed controls suggests that the pulmonary effects of vagotomy resemble phosgene poisoning[8,9] and leads to the conclusion that vagal efferent fibers to the lung and afferents from the lung, trachea and larynx have little, if any thing, to do with the pulmonary tissue's response to phosgene.[7] Unilateral vagotomy failed to produce detectable differences between homolateral and heterolateral lungs. These studies fail completely to confirm the claim of Laqueur and Magnus[1] that bilateral vagotomy before, but not after, gassing protects against phosgene poisoning.

Discussion. The bradycardia is not peculiar to phosgene poisoning since it has been noted in poisoning by chlorine, chlorpicrin and other lung irritants.[1,2,10] Presumably the same is true of the other cardiac signs of excessive parasympathetic tone. In man it has been reported to occur soon after gassing in combat,[1,11] but nevertheless it was not present in a large number of serious but nonlethal accidental exposures to phosgene.[12] Possibly among the latter the psychic response to being "gassed" may have contributed to the somewhat increased heart rates.

The cardiac reactions appear to be a reflex response. Vagotomy appears to sever only the efferent side of the arc as the other parasympathetic signs still occur. Therefore cranial nerves I, V, IX, parts of X and possibly sympathetic afferents[13] may serve as the afferent limb of the arc. Alternatively, there is produced in the tissues by action of phosgene a chemical which activates the parasympathetic centers or their specific afferents. While this humoral mechanism seems improbable to us (c.f.[6]), we are not able to suggest a complete explanation for the continued development of bradycardia *after* the stimulus, the phosgene, has been withdrawn.

Regardless of whether a state of exaggerated parasympathetic tone exists in the pulmonary as in the cardiac vagal fibers, the above data demonstrate that such innervation is not essential to the typical pulmonary reaction to phosgene. In particular, broncho- and bronchiolar constriction, if occurring at all,[7] is of no special significance.

Summary. By use of the electrocardiograph, vagotomy, and atropine, it was shown that the early bradycardia of experimental phosgene poisoning was mediated by efferent vagal fibers. Excessive parasympathetic tone appears to have no special significance in the genesis of the pulmonary response to phosgene.

---

[8] Coman, D. H., Bruner, H. D., Horn, R. C., Jr., Friedman, M., Boche, R. D., McCarthy, M. D., Gibbon, M. H., and Schultz, J., *Am. J. Path.*, 1947, **23**, 1037.

[9] Wieser, J., *Pflug. Arch. ges. Physiol.*, 1933, **321**, 68.

[10] Fleming, A. J., *Industrial Med.*, 1943, **12**, 127.

[11] Cole, H. H., *Am. Rev. Tuberc.*, 1923, **7**, 230.

[12] O'Connor, J. A., Personal communication, 1943.

[13] Miscall, L., and Rovenstine, E. A., *Surgery*, 1943, **13**, 495.

16456

Purified Human Poliomyelitis Virus: Infectivity for Cynomolgus Monkeys.*

CYRIL S. STULBERG, EBEN A. SLATER, HELENE P. BRUMFIELD, AND H. O. HALVORSON.
(Introduced by M. B. Visscher.)

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis.

In a series of studies on the purification of poliomyelitis viruses, it was of interest to determine the comparative activities of purified and unpurified human poliomyelitis virus. It was hoped that such an investigation would provide some information, however incomplete, on the approximate yield of active virus that might be expected from monkey spinal cord tissues by the methanol precipitation technique of purification.

It should be emphasized that the term *purified,* as used in this report, refers to preparations in which a relatively large proportion of protein has been removed, and by no means does it suggest that virus has been obtained which is completely free of non-viral protein.

Ultracentrifugal studies made by Loring and Schwerdt[1] showed that poliomyelitis-infected monkey tissues exhibited infectivity when diluted on a weight basis to 10^{-3}. After purification and concentration of the virus by ultracentrifugation methods, these workers demonstrated that preparations having 5×10^{-9} g of nitrogen per ml contained a minimal lethal dose of virus for rhesus monkeys.

In this investigation the comparative infectivity for cynomolgus monkeys of a recently isolated human poliomyelitis virus both before and after purification was studied. The virus was purified from nervous tissues by methanol precipitation according to a method described by Cox, *et al.*[2] for influenza viruses and modified by the authors for poliomyelitis viruses.[3]

During the 1946 poliomyelitis epidemic in Minnesota, spinal cords were collected from several human subjects who had exhibited spino-bulbar forms of the infection. After several months' storage in 50% buffered glycerine, 10% saline suspensions of these cords were inoculated intracerebrally into *Macacus rhesus* monkeys, which subsequently developed typical symptomatology and pathology of poliomyelitis. Spinal cord tissue from one of these monkeys was chosen for this study.

A second passage of the virus was made in a cynomolgus monkey by an intracerebral injection of 0.2 ml of a 10% saline suspension of spinal cord. This animal exhibited a marked temperature elevation and hyperexcitability on the sixth day after inoculation, and the next day developed extensive paralysis in all 4 limbs, at which time it was sacrificed. Histological examination of the brain and cervical and lumbar cord revealed typical lesions of a poliomyelitis infection. The entire spinal cord was removed aseptically and stored at –20°C until subjected to the purification procedures previously described.[3] Briefly, the technique used in the purification of infected monkey spinal cord necessitated preliminary grinding of the tissue, dilution in distilled water to form a 20% suspension, and two successive freezings and thawings with intermittent centrifugation of the heavy tissue sediments that formed. The slightly turbid supernate that resulted from the second centrifugation was arbitrarily called crude virus suspension. As shown in Table I, micro-Kjeldahl determination of the total nitrogen present in this suspension revealed

---

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Loring, H. S., and Schwerdt, C. E., *J. Exp. Med.*, 1942, **75**, 395.

[2] Cox, H. R., Van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

[3] Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 410.

TABLE I.
Infectivity of Purified and Crude Human Poliomyelitis Virus in Cynomolgus Monkeys.

	Mg N per ml	Dilutions				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Crude virus suspension	1.28	5/5*	3/3	2/3	0/3	1/3
Purified virus suspension	.0098	3/3	3/3	2/3	0/3	0/3

* Denominator indicates number of monkeys inoculated; numerator indicates number of infections.

1.28 mg of nitrogen per ml.

Twenty ml of the crude virus suspension was then treated with ether and centrifuged. The lipoidal layer was removed, the dissolved ether distilled off, and the aqueous suspension diluted to 200 ml with 0.1 molar phosphate buffer at pH 7. Absolute methanol cooled to –40°C was added to make a concentration of 30%, and the mixture was kept at –4°C for one hour. The resultant turbid suspension was centrifuged, the precipitate washed with 0.02 M phosphate buffer pH 5.6, and the undissolved precipitate redissolved in 20 ml of 0.1 M phosphate buffer pH 7. Subjection of the resultant water-clear suspension to micro-Kjeldahl procedure for total nitrogen determination revealed .0098 mg N per ml. Comparison of the nitrogen determinations on equal volumes of crude and purified suspensions showed that 99.2% of the nitrogen present in the crude suspension had been removed by methanol purification.

To determine the minimal infective dose of virus present in the crude suspension serial ten-fold dilutions to 10^{-5} were prepared, and each dilution was injected intracerebrally in dosages of 0.5 ml into cynomolgus monkeys. Since the animals were to be used subsequently in another contingent investigation, the conditions necessitated the use of five monkeys for the 1-10 dilution and groups of 3 monkeys for each of the higher dilutions.

Daily temperatures were obtained for each of the animals. The average rectal temperature of the monkeys, based on recordings made previous to the inoculations and during the first 3 days of the incubation period, was 103°F. A rise of 2 degrees was considered indicative of early symptoms. All infected animals exhibited at least a 3-degree rise before their temperatures dropped to a subnormal level upon the advent of paralytic symptoms. The incubation periods varied from 6 to 20 days.

Monkeys showing typical symptoms of poliomyelitis were sacrificed, and subsequent histological examination of their spinal cords confirmed the clinical diagnoses. All the animals inoculated with doses of crude virus below 10^{-3} developed infections, as did 2 of 3 in the 10^{-3} dilution group and one of 3 in the 10^{-5} dilution group. One of the animals of the 10^{-3} group recovered, although it exhibited residual paralysis in both legs. There were no indications of infection in the remaining monkeys. Although the error involved in monkey titration limits the calculation of an exact value for the minimal infective dose, it can be estimated from these results that approximately 1000 to 10,000 infectious units were present in the crude material. Protection against challenge inoculations or pathological evidence of infection could not be determined in the animals that did not show symptoms during this experiment. Results of the infectivity test and nitrogen determination are shown in Table I.

Titration of the purified product in cynomolgus monkeys disclosed a close correlation in the activity of crude and purified virus. In the latter titration two of the animals of the 10^{-3} dilution group developed typical symptoms and pathology of poliomyelitis, indicating that the estimated 1000 minimal infective doses in crude material were also present in the purified material.

During the course of a parallel investigation, an attempt was made to infect cynomolgus monkeys by the oral administration of crude and purified virus. Infectivity of strains of poliomyelitis viruses for several species of monkeys when administered by the oral or gastro-intestinal route has been reviewed by Horstmann and her co-workers,[4] who report successful transmission of these viruses when virus in the form of infected spinal cord or feces was injected.

In our experiments gelatin capsules were filled with 1 ml of a 10^{-1} dilution of crude and purified viruses. The capsules were placed in a balling gun commonly used in veterinary practice and injected into the esophagus. The capsules were then swallowed by the animals.

Crude virus suspension was administered in this manner to seven cynomolgus monkeys, and purified virus similarly to three cynomolgus monkeys. Two of the animals fed the crude virus developed paralytic symptoms of poliomyelitis, one in 11 days after the inoculation and the other in 18 days. The fact that the first monkey, which died from the infection, had accidentally bitten the capsule at the time of inoculation, may have resulted in infection by another route. Evidence of infection by another was not available, however, since examination of the olfactory bulbs or nerves was not made. In the case of the second monkey, no virus was allowed to come in contact with the tissues of the oropharynx, and it was assumed that the intact capsule passed into the stomach and that the resultant infection of the CNS was accomplished via the alimentary route. Of the 3 animals fed purified virus, one developed paralytic symptoms of poliomyelitis after an incubation period of 23 days. Histological examination of the cords of the three monkeys exhibiting symptoms confirmed the diagnoses.

Conclusions. Methanol precipitation at low temperatures resulted in partial purification of human poliomyelitis virus, in which a significant amount of protein was eliminated from infected monkey cord. Intracerebral titration in cynomolgus monkeys revealed that there was little observable loss of infectious units of virus during the purification procedure. It was observed that approximately 5 x 10^{-9} g of N injected into monkeys provided a minimal lethal dose, although considerable error may be involved since this value was dependent on the highly variable monkey titration. However, this value indicates a remarkable correlation with that reported by Loring and Schwerdt,[1] although the nitrogen value for methanol-precipitated virus is lower than the values obtained when virus was purified by ultracentrifugation methods.

Infected monkey cord exhibited a rather low infectivity for cynomolgus monkeys when administered orally by capsule. It is interesting to note that in one instance the purified virus was infective and that apparently this infectivity did not differ appreciably from that of the crude virus when inoculated orally. Other investigators[5] have been unsuccessful in infecting cynomolgus monkeys by feeding virus contained in gelatin capsules.

Summary. Human poliomyelitis virus in the form of infected monkey cord was purified by methanol precipitation at low temperatures. Titration by intracerebral inoculation in cynomolgus monkeys indicated no appreciable loss in activity of the virus during the purification procedure. It was shown that purified virus was infective for cynomolgus monkeys when administered by the oral route.

The authors wish to thank Dr. A. B. Baker for examining the histological sections.

---

[4] Horstmann, D. M., Melnick, J. L., Ward, R., and Sa Fleitas, M. J., *J. Exp. Med.*, 1947, **86**, 309.

[5] Faber, H. K., Silverberg, R. J., and Dong, L., *J. Exp. Med.*, 1943, **78**, 499.

16457

Nonglucose Reducing Substances in Hypertension.

HAROLD JOSEPH AND LOUIS TOBIAN, JR.* (Introduced by T. R. Harrison.)
From the Department of Internal Medicine, Southwestern Medical College, Dallas, Texas.

Although the blood of nonuremic patients with essential hypertension has shown few chemical abnormalities, there have been reports of increased nonglucose reducing substances.[1] Since these nonglucose reducing substances could conceivably have a blood pressure raising effect, they were determined in the whole blood and plasma of normal and hypertensive individuals.

Procedure. The blood pressures were taken by the usual cuff method, the diastolic pressure being that point at which the pulse sound disappeared, as recommended by Steele.[2]

Blood was drawn under fasting conditions into bottles containing sodium citrate, and the determinations were made immediately.

The analytical method employed was a modification of Somogyi's method for the determination of reducing nonsugars in blood.[3] Glucose was removed by washed baker's starch-free yeast prepared according to Somogyi.[3] Sodium hydroxide and zinc sulfate were used to precipitate the protein in whole blood.[4] Colorimetric determinations were made according to Somogyi,[4] using his new alkaline copper reagent[5] and Nelson's arsenomolybdate reagent.[6] A glucose standard was analyzed with each determination. All samples were read on an Evelyn colorimeter, using the 660 Evelyn filter.

The nonglucose reducing substances were also determined in plasma by an identical

TABLE I.
Observations on Protein-free Filtrates of Whole Blood and Plasma of Normal and Hypertensive Subjects.

	No. of samples	Range of blood pressures (mm Hg)	Mean of blood pressures (mm Hg)	Mean of nonglucose reducing substances estimated as glucose mg %	S.E. of means	Diff. in means S.E. of diff. in means
A. Whole blood:						
Normal	19	138-98 80-48	106 63	4.14	.399	1.07
Hypertensive	20	250-142 128-96	181 107	4.87	.555	(P = .28)
B. Plasma:						
Normal	10	138-100 80-60	119 73	1.46	.312	.36
Hypertensive	11	240-142 118-98	182 109	1.76	.269	(P = .72)

---

* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Holden, Raymond, F., Jr., *J. Clin. Invest.*, 1944, **23**, 859.

[2] Steele, J. M., *J. Mt. Sinai Hosp.*, 1942, **8**, 1042.

[3] Somogyi, Michael, *J. Biol. Chem.*, 1927, **75**, 33.

[4] Somogyi, Michael, *J. Biol. Chem.*, 1945, **160**, 69.

[5] Somogyi, Michael, *J. Biol. Chem.*, 1945, **160**, 61.

[6] Nelson, Norton, *J. Biol. Chem.*, 1944, **153**, 375.

method except that barium hydroxide was substituted for sodium hydroxide in the protein precipitation.[4]

Values for nonfermentable reducing substance have been expressed in terms of glucose-equivalents and have, therefore, only relative significance.

Results. The results of whole blood and plasma determinations of nonglucose reducing substances are shown in Table I, as well as the standard error of their means. The difference between the levels of nonglucose reducing substances of normal and hypertensive subjects, when subjected to statistical analysis, cannot be regarded as significant. The variance between our results and those of Holden[1] can possibly be accounted for by the fact that he was using Somogyi's earlier copper reagent which employs a carbonate-bicarbonate buffer mixture and contains KI. The reagent we used contains a phosphate buffer system and no KI.

Summary and Conclusions. The nonglucose reducing substances remaining after a Somogyi protein precipitation[4] were determined in blood and plasma.

The blood or plasma concentration of these nonglucose reducing substances was not significantly different in hypertensive subjects as compared to normals.

16458 P

Effect of Adrenal Cortical Extract in Hypertensive Subjects.*

Kermit L. Pines, George A. Perera, Katherine Vislocky, and Ann D. Barrows.
From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital, New York City.

Following studies on the pressor activity of desoxycorticosterone acetate,[1] suppression of adrenal cortical function was considered as a possible means of decreasing the blood pressure in essential hypertension. Observations were therefore undertaken on the effects of prolonged administration of an adrenal cortical extract on the arterial tension, circulatory physiology, electrolyte and fluid balance of patients with this disorder.

Methods. Four women with uncomplicated hypertensive vascular disease were studied on the metabolism ward of the Presbyterian Hospital. The criteria in selection of patients and the methods employed were identical to those previously described.[2] All subjects were maintained on a constant dietary fluid and sodium chloride intake and were afebrile throughout the experiment. Following a 3-week baseline period, adrenal cortical extract (Upjohn) was administered intramuscularly in 10 cc doses twice daily for 20 days; then, in 3 patients, 3 times daily for an additional 10 days. The remaining patient (No. 3) developed a moderately severe headache after 6 days on the increased dosage so that the schedule of 2 injections was resumed for 9 additional days. During the final week of the preliminary period and for one week after the extract was discontinued, each patient received intramuscular injections of glucose in distilled water or of 10% alcohol twice daily as well as the advice that this medication was producing a beneficial effect.

Results. Slight weight gain relative to the baseline trend appeared during the period of adrenal cortical extract administration in all instances, together with minimal evidence of salt and water retention. In 3 subjects a decrease in "resting"[2] blood pressure was observed, with a rapid return to baseline values

---

* Aided by grants from the National Institute of Health (U.S.P.H.S.) and the Albert and Mary Lasker Foundation.

[1] Perera, G. A., and Blood, D. W., *Ann. Int. Med.*, 1947, **27**, 401.

[2] Perera, G. A., and Blood, D. W., *J. Clin. Invest.*, 1947, **26**, 1109.

TABLE I.
Effects of Adrenal Cortical Extract on Blood Pressure and Laboratory Data in 4 Patients with Hypertensive Vascular Disease.

	Patient	Wk before ACE	Final wk of ACE	Wk after ACE
Mean B.P. (mm Hg)	1	169/95	151/81	175/93
	2	183/109	155/91	174/105
	3	182/100	167/90	181/100
	4	167/78	173/82	157/78
Min., Max. Systolic and Diastolic Values	1	$\frac{156-180}{88-108}$	$\frac{130-160}{76-90}$	$\frac{164-190}{86-100}$
	2	$\frac{172-198}{104-118}$	$\frac{146-166}{84-96}$	$\frac{166-180}{98-114}$
	3	$\frac{170-190}{94-108}$	$\frac{158-180}{86-96}$	$\frac{170-196}{94-104}$
	4	$\frac{158-176}{74-82}$	$\frac{164-180}{78-84}$	$\frac{146-170}{70-84}$
Serum Sodium, meq./l	1	139.6	137.0	140.4
	2	138.6	142.1	139.1
	3	142.4	141.8	141.0
	4	143.5	142.4	144.0
Serum Potassium, meq/l	1	4.1	3.6	4.1
	2	5.2	4.5	4.8
	3	4.7	4.3	4.6
	4	4.5	4.7	4.2
Serum Chlorides, meq/l	1	100.9	97.2	104.1
	3	97.8	101.4	101.6
	4	102.0	97.8	104.6
Serum CO_2 Content, meq./l	1	28.2	30.3	28.7
	3	32.1	29.5	29.6
	4	28.9	30.1	27.5
Urine Sodium, meq./24 hrs	1	48	45	46
	3	43	49	56
	4	38	55	60
Urine Potassium, meq./24 hrs	1	65	63	53
	3	65	59	58
	4	41	45	51
Urine Chlorides, meq./24 hrs	1	45	44	66
	3	64	59	63
	4	42	58	55
Serum Proteins, g/100 cc	1	7.4	7.3	7.3
	2	7.5	7.2	7.5
	3	6.5	6.9	6.8
	4	7.0	6.5	6.4
Serum Vol., cc	1	2530	2640	—
	2	3220	3300	—

during the final week of placebo therapy. A small increase in blood pressure took place in one patient (No. 4) in whom "resting" diastolic values had fallen to normal limits shortly after hospital admission (Table I).

Decreases in serum potassium levels (of 0.7

milliequivalents per liter or less) were noted in 3 cases, but no significant change could be detected in hematocrit, carbon dioxide content, chloride, sodium, protein or urea nitrogen concentration of the serum. There was no major alteration in the excretion of sodium, potassium or chloride in the urine. Determinations of serum volume with the blue dye T. 1824 were carried out in 2 subjects before and at the end of the period of extract injection with no significant change. Ballistocardiographic tracings were unaltered in one patient who exhibited a drop in blood pressure. Toward the close of the extract period there was no leucocytosis, eosinophilia or rise in erythrocyte sedimentation rate; no effect on the electrocardiogram or teleroentgenogram; and the patients complained of no symptoms during the treatment period other than the headache previously mentioned.

Discussion. In the dosage employed, the continued administration of adrenal cortical extract was associated with a decrease in "resting" blood pressure in 3 of 4 hypertensive patients. The changes in arterial tension could not be ascribed to alterations in serum volume, fluid or electrolytes, or apparent change in cardiac action.

The results suggest the possibilities that the fall in blood pressure may reflect suppression of adrenal cortical function or represent counteraction of a desoxycorticosterone-like pressor hormone. The adrenal cortex may possess a homeostatic group of substances concerned with blood pressure regulation.[3] However, one cannot exclude the possibility that the decrease in tension observed in this study may have been the result of some non-specific component contained in the extract. Further search for depressor materials is indicated among purified products of the adrenal cortex.

Summary. The continued administration of an adrenal cortical extract for one month, to four subjects with uncomplicated hypertensive vascular disease on a constant regimen, was associated with a decrease in "resting" blood pressure in 3 instances, a small rise in arterial tension in one.

---

[3] Soffer, L. J., *Diseases of the Adrenals*, Lea & Febiger, Philadelphia, 1946.

16459

Influence of pH on Growth of Bone in Tissue Culture.*

GEORGE H. PAFF.
From the Department of Anatomy, Long Island College of Medicine, Brooklyn, N.Y.

Although a great amount of work has been done on the general subject of bone growth, the exact actions of the factors involved are poorly understood. The problem of bone growth and pH is a case in point. With little or no experimental evidence the idea is widespread that the calcification which accompanies bone growth occurs because of a local change to a more alkaline pH. To ascertain whether or not this speculation was correct we undertook to grow bones in tissue culture at two levels of pH, namely 7.0-7.3 and 7.8-8.0.

The pertinent literature on the problem traces largely from the work of Robison and his co-workers. Following Robison's discovery[1] of phosphatase in bone the theory was advanced that this enzyme played an important role in ossification. Further work revealed that in the test tube phosphatase was most active between pH 8.4 and 9.4 when glycerophosphate was employed as the substrate.[2] Robison then postulated that osteo-

---

* Supported by a grant from the United States Public Health Service. The author is also indebted to Miss Marie T. Coppola for her technical assistance.

[1] Robison, R., *Biochem. J.*, 1923, **17**, 286.

[2] Robison, R., and Soames, K. M., *Biochem. J.*, 1924, **18**, 740.

blasts and hypertrophic cartilage cells could raise the pH of tissue fluid around them. The effect of this on a saturated solution of $Ca_3(PO_4)_2$ and $CaCO_3$ would be to produce precipitation of the salts. However it was not considered essential that the pH be raised as high as 8.4; a small change would cause the desired effect.[3]

The above findings and opinions were used by Fell[4] to explain, in part, the slowness of ossification in tissue culture as compared with the greater speed of the process *in vivo* since the average pH of the medium in which tissue is growing is more acidic than is the pH of normal blood plasma.

Others have subscribed to the theory[5,6] and even extend it to explain calcification in degenerating cartilage. As chondrocytes degenerate, metabolic activity drops, fewer metabolites are produced, the pH rises and calcification is thereby favored.[6]

Howland[7] was more specific. He visualized the calcification process involved in bone growth as depending on CO_2 tension. The presence of a relatively high CO_2 level due for example to cellular activity is unfavorable to deposition of bone salts but in an inactive tissue such as bone and cartilage "one would expect the CO_2 tension to be low and it is in them that precipitation takes place."

Direct experimental observations do not support the idea that a local increase in pH favors bone formation.[8] The early work of Rous[9] with indicators injected into the living animal revealed pH's in bone of 6.5 to 7.2. Pierce[10] made direct observations on the pH of epiphyseal cartilage in tibias of rachitic and normal rats. The bones were split and laid open like a book. Using a special quinhydrone electrode mounted on a micromanipulator, he obtained no detectable pH differences between resting and proliferating cartilage. Studies[11] on the ion products of calcium phosphates in normal serum with changes in pH led to the conclusion that any serum which does not cause calcification (presumably of cartilage slices) at the pH of the blood will not do so when the pH is raised to 8.0.

As regards the cultivation of bone in tissue culture it seems almost superfluous to mention the excellent and widely quoted works of Fell.[4,12,13]

Method. In growing bones in tissue culture a technic modified after that of Fell[14] was used. Vials that held 32 cc were used instead of Petri dishes and watch glasses. Each vial received 15 drops of Tyrode solution containing phenol red, 15 drops of embryonic chick extract and 15 drops of blood plasma. After the media were thoroughly mixed the vials were laid horizontally and left unmolested while the clot formed. The vials were taped to a U-shaped piece of metal rod to prevent rolling.

Femurs were dissected from 7 day chick embryos and those from one animal placed in marked areas of separate vials (Fig. 1). Routinely 4 femurs were put in each container. In each experiment a minimum of 16 femurs were used.

The pH of the media was controlled by the continuous flow of gases through the vials. A two-holed paraffined cork supporting an inlet and an outlet tube was employed instead of the usual blind cork stopper (Fig. 1). Each tube had a cotton filter. A continuous stream of the desired gas passed into the vial through one tube and out of the other. Two vials received air with no CO_2 at the rate of about one liter of gas for each vial per day. The pH of these vials as read colorimetrically against standard tubes was 7.8-8.0. Another two

---

[3] Robison, R., *Biochem. J.*, 1926, **20**, 388.

[4] Fell, H. B., *Arch. f. Exp. Zellforsch.*, 1929, **7**, 390.

[5] Howard, J. E., Conference on Bone and Wound Healing, 1st Meeting, p. 26, N.Y., 1942 (Josiah Macy, Jr., Found.).

[6] Weinman, J. P., and Sicher, H., *Bone and Bones*, pp. 40-43, C. V. Mosby Co., St. Louis, 1947.

[7] Howland, J., *Medicine*, 1923, **2**, 349.

[8] McLean, F. C., *Ann. Rev. Physiol.*, 1943, **5**, 79.

[9] Rous, P., *J. Exp. Med.*, 1925, **41**, 739.

[10] Pierce, J. A., *J. Biol. Chem.*, 1938, **124**, 115.

[11] McLean, F. C., Conference on Bone and Wound Healing, 14th Meeting, pp. 9-20, New York, N.Y., 1946 (Josiah Macy, Jr., Found.).

[12] Fell, H. B., *Arch. f. Exp. Zellforsch.*, 1931, **11**, 245.

[13] Fell, H. B., *J. Morph. and Physiol.*, 1925, **40**, 417.

[14] Fell, H. B., *J. Roy. Mic. Soc.*, 1940, **60**, 95.

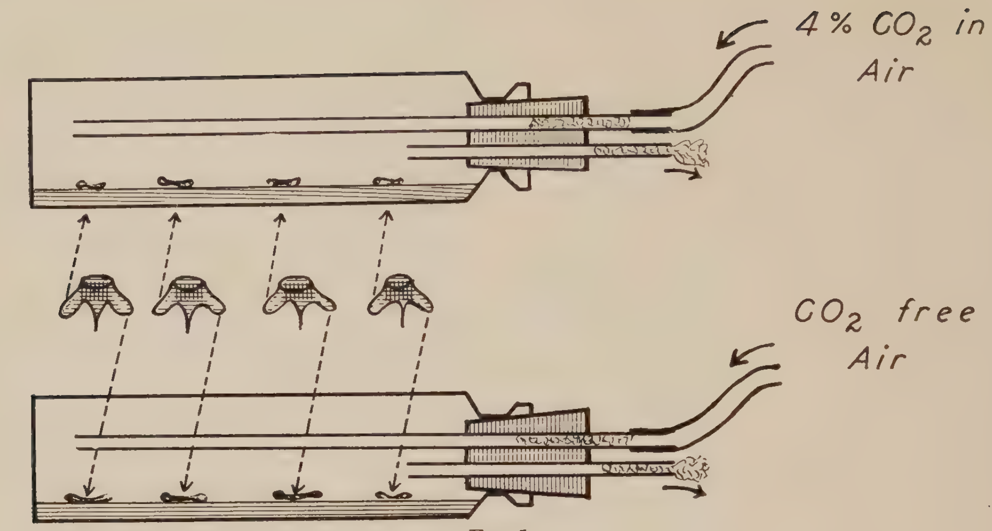


FIG. 1.

vials containing mates to the femurs in the first were flushed continuously with air containing 4% of CO_2, one liter per vial each day. The pH of these was 7.0-7.3 as read colorimetrically.

The femurs were transplanted every second or third day as deemed necessary. Cultivation in every case was arbitrarily terminated after 14 days. At this time 8 pairs of femurs were fixed in formaldehyde, embedded in paraffin, sectioned serially and stained with hematoxylin and eosin. Projection drawings were made of the areas in which bone had formed. Each bone was projected at the same magnification, and the relative amount of bone grown in each femur was determined by the paper weight method. In a second group of femurs the calcium was determined quantitatively. The femurs were wet ashed with sulfuric acid and 30% H_2O_2, neutralized, made up to a volume of 2 cc, and the calcium determined on aliquots by the method of Sobel and Sobel.[15]

Observations. Embryonic femurs growing in tissue culture look like femurs. Growth is organized. When planted at the 7th day of embryonic development the femurs consist entirely of cartilage and are quite small. At the end of 14 days of cultivation in the vials there has been a marked increase in size. In terms of normal development, however, the amount of growth occurring in 14 days of cultivation is equivalent to a scant 3 days of normal growth in the embryo.

As seen in section the cultivated femurs consist principally of cartilage with a well-formed zone of bone around the shaft. In hematoxylin-eosin preparations the bone appears as a distinct red band.

In Table I the relative amounts of bone

TABLE I.
The Relative Amounts of Bone Grown in 11 Pairs of Femurs. One of each pair was grown at pH 7.8-8.0; the other at pH 7.0-7.3. The amount of bone is expressed in terms of per cent. Bone growth at pH 7.8-8.0 was 100% in every test.

Tubes	Pair of femurs	Bone grown at pH 7.8-8.0	Bone grown at pH 7.0-7.3
1 and 2	1	100	111
	2	100	128
	3	100	90
	4	—	—
3 and 4	5	100	209
	6	100	134
	7	100	92
	8	100	197
5 and 6	9	100	171
	10	100	96
	11	100	113
	12	100	183
Avg		100	139

---

[15] Sobel, A. E., and Sobel, B. A., *J. Biol. Chem.*, 1939, **129**, 721.

TABLE II.
The Calcium Content of 8 Pairs of Femurs. One of each pair was grown at pH 7.8-8.0; the other at pH 7.0-7.3.

Tubes	Pair of femurs	Calcium in femurs grown at	
		pH 7.8-8.0	pH 7.0-7.3
A and B	1	43 γ	170 γ
	2	67 γ	54 γ
	3	63 γ	233 γ
	4	18 γ	116 γ
C and D	5	74 γ	166 γ
	6	46 γ	61 γ
	7	61 γ	98 γ
	8	39 γ	79 γ
Avg		51 γ	122 γ

expressed as per cent are shown for each member of 11 pairs of 7 day femurs after 14 days of growth in tissue culture. No data are presented for the fourth pair because one of them was accidentally fractured in transplanting to fresh media. Of the remaining 11 pairs of femurs it will be seen that in 8 of them the ones grown at pH 7.0-7.3 had distinctly more bone than their mates grown at the more alkaline pH of 7.8-8.0. Only 3 of the 11 pairs of femurs grown in the more alkaline medium contained more bone than did those grown at pH 7.0-7.3. The averages reveal that at a pH of 7.0-7.3 the amount of bone was approximately 40% more than at the more alkaline pH of 7.8-8.0.

In Table II the total amount of calcium present in each member of 8 pairs of femurs is listed. In 7 of them the bones growing at pH 7.0-7.3 contained distinctly more calcium than did their respective mates growing at the more alkaline pH of 7.8-8.0. In only one pair, the second, was there more calcium deposited at pH 7.8-8.0 than at 7.0-7.3. The average values indicate a marked increase in calcium in those femurs cultivated at pH 7.0-7.3.

Discussion. Before discussing the above observations let us recall what is meant by "the second calcifying mechanism" or "the local factor." According to the phosphatase theory of calcification the enzyme acts on organic phosphates to liberate phosphate ions which react in turn with calcium ions to produce a form of calcium phosphate. The calcium phosphate is deposited in areas where ossification is occurring. But why does not calcification occur in all places where phosphatase, organic phosphates and calcium ions are found, as *e.g.*, in the wall of the intestine? It is thought that a second mechanism, some local factor, must be present before calcification will occur. Such a factor is not present in the intestinal wall but is found in areas where bone formation is progressing. There are various theories as to just what the local factor is. As indicated above many people regard it as being a local change toward a more alkaline pH.

Our results, however, are not in agreement with the concept that bone formation in a given area is favored by a local change in pH toward the alkaline. The pH of chicken blood is about 7.6 yet when the femurs are grown at a pH of 7.0-7.3 about 40% more bone is found on the average than when the femurs are held at a pH of 7.8-8.0. In addition the calcium determinations reveal that the average calcium content of femurs grown at pH 7.0-7.3 was 122γ but only 51γ in femurs grown at pH 7.8-8.0. For 2 weeks the femurs lay on the surface of a clot and were exposed directly to the atmosphere which controlled the pH. Considering that the bones were isolated it seems reasonable to assume that as regards pH the local factor was well controlled. Bearing these facts in mind the statement is entirely justified that an increase in alkalinity from 7.0-7.3 to 7.8-8.0 favors neither bone formation nor calcium deposition in bones growing in tissue culture.

Objections may arise on the basis that the paper weight method of determining the amount of bone is crude. The method is admittedly not very accurate in that we are dealing with small amounts of bone and the subjective element is a formidable one. The method is however a practical one and was used to distinguish the amount of bone formation and calcification. The bone is distinguishable in the stained preparations as a red zone bearing the same positional relationship to the underlying cartilage as a napkin ring to the napkin. While the 39%

average difference may not be accurate, a difference in the amount of bone existed and a smaller amount was found in the more alkaline medium.

The values for calcium need qualification. It is not implied that they represent only the calcium deposited in the bone. Instead the entire amount of calcium deposited throughout the life of the femurs, *i.e.*, 7 days *in ovo* and the additional 14 days of cultivation in tissue culture, was measured. Moreover, the calcified area is not restricted to the zone of ossification, but involves portions of the cartilage as well. Yet, based on the assumption that one femur contains the same amount of calcium as its mate when first planted in tissue culture, the statement is entirely reasonable that the difference in the calcium values for each member of the 8 pairs listed in Table II is due to variations in pH over the 2 weeks of cultivation *in vitro*.

The quantity of calcium and phosphate available in the media upon which the bones grew was not determined. It was presumed to be adequate since the media consisted of adult chicken blood plasma, embryo extract made from 10 to 11 day chick embryos and Tyrode solution. The precaution was taken to use the same stock media for both the low and high pH cultures.

The most intriguing part of the problem concerns the enzyme phosphatase. From the work of Fell and Robison[16] it is known that not only is it synthesized in the bones grown in tissue culture but it is present in amounts larger per unit weight than can be found in bones *in vivo*. Phosphatase is the key component of the phosphatase theory of bone formation. In the hands of the chemist[17] the activity of the enzyme "rises rapidly from pH 7.0 to pH 9.0: *e.g.* at pH 7.5 the activity is double that at pH 7.0 and at pH 8.0 more than fourfold the value at pH 7.0." If it is assumed that the amount of the bone formed and the amount of calcium deposited is an indication of phosphatase activity then why is less bone formed at a high pH than at a low one? At a pH of 7.8-8.0 the enzyme should be from 2 to 4 times as active as at a pH of 7.0-7.3.

While we do not agree with the implied doubt of Shipley, Kramer and Howland[18] and later McLean[11] that phosphatase plays a key role in the calcifying mechanism, we did not expect the results which we obtained. The pH 7.8-8.0 is one which will support general growth of embryonic chick tissue very well (Paff).[19] From the day by day appearance of the bones growing in culture one could not observe any gross difference between femurs grown at a low and at a high pH, yet they did differ. Perhaps the activity pattern of phosphatase in the living tissue differs from the pattern followed by the enzyme in the simpler systems not involving live tissue.

The observations are conclusively in favor of discarding the idea that the local factor in bone growth is a change in pH toward a more alkaline reaction. In bone growing in tissue culture it can be said that if a change in pH is desirable then it is in the direction of less, not more, alkalinity. By decreasing the pH perhaps more nonionized calcium is ionized making it more readily available for reaction with phosphate. Since PO_4 ions are more numerous at the site of phosphatase activity, *i.e.*, in areas where osteoblasts are found, it should be in this region that more bone would be produced.

Our results are the exact opposite of those which would be predicted on the basis of Howland's CO_2 theory.[8]

Summary and Conclusions. Seven day chick embryo femurs were grown in tissue culture for 2 weeks at 2 pH levels, *i.e.*, pH 7.0-7.3 and 7.8-8.0. The pH's were maintained by a continuous flow of gas, 4% CO_2 in air for the low level and air minus CO_2 for the high. Our results show that more bone is formed and more calcium is deposited at the low than at the high pH. If a local change in pH is desirable in bone growing in tissue culture then it is in the direction of less rather than more alkalinity. These data are strong evidence against the widespread belief that the local factor in bone growth is a change in pH toward the alkaline.

---

[16] Fell, H. B., and Robison, R., *Biochem. J.*, 1929, **23**, 767.

[17] Borne, G., *Cytology and Cell Physiology*, p. 219, Oxford Univ. Press, 1941.

[18] Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, **26**, 379.

[19] Paff, G. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 184.

16460

Response of the Syrian Hamster to the Virus of Newcastle Disease.

Reginald L. Reagan, Mary G. Lillie, Jean E. Hauser, and A. L. Brueckner.

From the Maryland State Board of Agriculture, University of Maryland, College Park, Md.

The adaptation of the California strain of Newcastle disease virus No. 11,914 to the Syrian hamster through 12 intracerebral passages has been reported.[1] This Newcastle virus has been passed through 22 successive series of 10- to 12-day embryonated chicken eggs prior to hamster injection. Intracerebral culture in hamsters has been continued through 200 uninterrupted passages.

Infected hamsters used for passage were sacrificed while moribund. Suspensions were prepared by grinding the infected brain tissue in a mortar with alundum and diluting with physiological saline. The inoculum for the first 50 successive passages was approximately 0.08 cc of a 10% (10^{-1}) suspension; for the 51st to the 120th passage 0.03 cc of suspension of the same concentration was used; and after the 150th passage each hamster was inoculated with 0.03 cc of a 1% (10^{-2}) suspension.

The incubation period in the first 12 passages extended from 2 to 6 days. In these early passages symptoms of irritability followed by paralysis developed slowly over a period of approximately 2 days, and these hamsters remained alive for at least one day after becoming paralyzed. Approximately 50% of the inoculated hamsters of the first 8 passages showed irritability and increased sensitivity, followed by involuntary motor reactions and paralysis, before becoming moribund. After the eighth passage all inoculated hamsters developed the above-mentioned typical signs of central nervous system involvement.

From the 70th through the 120th passages, signs of central nervous system involvement were observed approximately 36 hours following inoculation, and most of the hamsters became moribund in 48 hours. From the 160th passage through the 190th passage the hamsters showed signs of central nervous system involvement in 24 hours and died within 36 hours of the inoculation. After the 190th passage infected hamsters showed signs of central nervous system involvement in 12 hours and 75% died within 24 hours.

Hamster brain suspensions from the 49th and 72nd passages titrated 10^{-3} by intracerebral inoculation in 4-week-old hamsters. The infected brain suspensions of the 200th passage titrated $10^{-4.25}$ in 4-week-old hamsters and $10^{-3.5}$ in adult hamsters, as is shown in the table.

Neither the egg-adapted nor the hamster-adapted Newcastle disease virus produced symptoms of disease in hamsters when injected subcutaneously, intramuscularly, intradermally, or intraperitoneally.[2] The hamster-adapted Newcastle disease virus after the 92nd passage was not pathogenic for chickens between 3 and 6 weeks of age when injected intramuscularly or subcutaneously.

---

[1] Reagan, Reginald L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

[2] Reagan, Reginald L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Titration of the Hamster-Adapted Newcastle Virus of the 200th Passage.

Hamsters Age in weeks	Dilutions						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer[3]
4	5/5	3/3	4/5	2/2	0/6	0/6	$10^{-4.25}$
12	5/5	4/4	2/2	0/6	0/5	0/5	$10^{-3.5}$

Numerator—Number of deaths. Denominator—Total number inoculated.

Summary. A California strain of Newcastle virus (No. 11,914) was carried serially by intracerebral inoculation through 200 passages in Syrian hamsters. The virus produced symptoms of irritability followed by involuntary motor reactions and paralysis. During the state of paralysis and while moribund, a large percentage of the infected hamsters showed marked labored breathing. The incubation period decreased gradually from 2 to 6 days in the early passages to approximately 12 hours after the 190th passage. Despite the apparent increased infectivity with progressive passages, the hamster-adapted Newcastle virus of the 200th passage titered only slightly higher in 4-week-old hamsters than that of the 49th or 72nd passages.

After the 92nd passage the hamster-adapted Newcastle disease virus was non-pathogenic for chickens when inoculated intramuscularly or subcutaneously.

16461

Artificial Enrichment of Beet Root Tissue with Glutamine.

HUBERT BRADFORD VICKERY, GEORGE W. PUCHER,* AND CHARLES S. LEAVENWORTH.

From the Connecticut Agricultural Experiment Station, New Haven, Conn.

Current interest in glutamine as a metabolite in animal tissues has led to a demand for this substance for experimental purposes. Glutamine may be prepared in quantity from beet root tissue by a method modified from the original procedure of Schulze and Bosshard[1] by Vickery, Pucher and Clark[2] but the yield obtained is dependent upon the richness of the material employed and this may vary from about 2 g per kg of trimmed root tissue to as much as 5 g. However, samples that contain more than 3 g per kg are not common.

It was observed[3] some years ago that the roots become notably enriched in glutamine if beet plants are treated in the field with 2 M ammonium sulfate solution at the rate of approximately a ton of nitrogen per acre (37 liters of solution per 100 square feet), the plants being collected 4 to 10 days later. Under these circumstances, the glutamine content increased from less than 2 to about 3 g per kg. Beet plants growing in the greenhouse and treated daily for 18 days with 0.5 M ammonium sulfate in such a way that the cumulative supply of nitrogen was somewhat greater than was used in the field experiment yielded roots that contained as much as 8 g per kg of glutamine or the equivalent of 5.4% of the dry weight. However, the opportunity to prepare beet plants in this manner for glutamine isolation is seldom available to workers in biochemical laboratories, and it

---

* Died November 20, 1947.

[1] Schulze, E., and Bosshard, E., *Ber. chem. Ges.*, 1883, **16**, 312.

[2] Vickery, H. B., Pucher, G. W., and Clark, H. E., *J. Biol. Chem.*, 1935, **109**, 39.

[3] Vickery, H. B., Pucher, G. W., and Clark, H. E., *Plant Physiol.*, 1936, **11**, 413.

TABLE I.
Enrichment of Beet Root Tissue in Glutamine by Culture of Plants for 138 Hours in 0.15 M Ammonium Sulfate.

Method of preparation for analysis	Before culture		After culture	
	Dried	Extracted	Dried	Extracted
Fresh wt root tissue, g	350	420	371	399
Dry wt, g	62.4		58.7	
Ammonia N, g/1000 g fresh wt	0.150	0.018	0.797	0.787
Glutamine, g/1000 g fresh wt	3.15	4.10	4.20	5.24
Asparagine, g/1000 g fresh wt	0.47	0.87	0.85	0.47
Loss of glutamine on drying tissue, g	0.95		1.04	
Loss of glutamine on drying tissue, %	23.2		19.8	
Increase of glutamine on culture, g/1000 g fresh wt			1.05	1.14
Increase of glutamine on culture, %			33	28

therefore seemed desirable to devise a method of treatment that could be applied to the plants as purchased at the market, and at any time of the year that they are available.

After preliminary experimentation on the concentration of ammonium sulfate solution and the time necessary to produce worthwhile effects, 24 beet plants selected for uniformity in size and development from a lot obtained in the local market were sorted into 2 equal groups one of which was put into an enamel ware pail that contained 3 liters of 0.15 M ammonium sulfate solution so that the roots were immersed and the tops supported by the walls of the pail. This sample was then placed in the greenhouse where it remained for 6 days. Presumably, any sunny place would have been equally suitable.

The second sample, to be used as a control, was trimmed of leaves and small fibrous roots and the main root was cut lengthwise in half to provide 2 similar subsamples. One of the portions of half roots was then sliced thin and dried at 80°C in a ventilated oven until crisp and was ground to powder in a Wiley mill. The other portion was ground in a meat chopper, the mass was treated with sufficient ether to saturate the aqueous phase in order to plasmolyze the cells and, after 30 minutes, was enveloped in drilling and pressed between steel plates in the hydraulic press. The press cake was shredded, thoroughly moistened with water and pressed again, and this washing operation was repeated a second time. The total volume of extract and washings was 1000 ml. The extract was immediately analyzed for ammonia and for glutamine and asparagine amide nitrogen. The same analytical determinations were made on the dried control sample.

At the end of the period of culture in ammonium sulfate, the other lot of beet plants was treated in the same way save that the roots were thoroughly washed to remove culture solution before being trimmed and prepared for analysis. The data are given in Table I and support the following conclusions. The glutamine content of beet root tissue can be appreciably increased by culture of the whole plant in 0.15 M ammonium sulfate solution for approximately a week even when, as in the present case, the initial glutamine content of the tissue was unusually high. The apparent glutamine content of beet root tissue is seriously diminished if the tissue is dried at 80°C, doubtless because of the instability of glutamine in the presence of water at elevated temperatures. Nevertheless, if the samples before and after treatment are prepared for analysis in the same way, an equally valid demonstration of the enrichment is provided, whether the material is dried in the oven or extracted with water at room temperature. Ammonia is freely taken up by the roots under the conditions described and is, in part, employed for the synthesis of glutamine. However, no significant increase in asparagine was observed.

The presence of the leaf tissue is essential for the reaction. A similar experiment gave evidence for an increase from 2.4 g per kg of fresh root tissue in the control to 4.3 g in the roots of the plants with leaves attached which were treated with 0.15 M ammonium sulfate for 98 hours. On the other hand, when trimmed roots from the same lot were subjected to the same conditions, no significant change in glutamine was noted in spite of a clearly demonstrable increase in ammonia. The data for this experiment were obtained from the analysis of dried samples and doubtless represent an underestimate of the actual quantities of glutamine present.

It should perhaps be pointed out that experiments of this kind with field grown beet plants are frequently unsatisfactory owing to the inhomogeneity of the material with respect to glutamine content. When small lots are employed, the most careful selection of plants for apparent similarity may fail to give samples that duplicate each other sufficiently closely for reliable results. However, the experiment has been repeated a number of times and consistent behavior observed. Although the data given in detail in Table I are from an experiment that was by no means as striking with respect to the magnitude of the increase in glutamine as was observed in some of the other trials, they furnish the clearest evidence that has yet been presented of the course of the phenomenon and demonstrate that the effect of *in vitro* culture of beet roots with tops attached in ammonium salt solutions follows the same pattern after the plants have been removed from the soil as it does in undisturbed plants in the field. Furthermore, it is clear that treatment in this way of beet plants that are to be used for the preparation of glutamine in quantity is greatly to be desired if high yields are sought.

Special emphasis should perhaps be given to the point that determinations of glutamine amide nitrogen in beet root tissue that has been subjected to drying at 80°C are unreliable as an index of the glutamine content of the fresh tissue because of the danger of hydrolysis of a part of the glutamine during the drying process. It is probable that earlier published data obtained in this way are somewhat low.

Summary. The glutamine content of beet root tissue to be used for the preparation of glutamine on the large scale can be materially increased if the roots of the plants with tops attached as purchased in the market are immersed in 0.15 M ammonium sulfate solution for from 4 to 6 days before being trimmed and extracted with water. Tissue that will yield 5 g or more of glutamine per kg can be prepared in this way. A demonstration is afforded that, contrary to earlier observations, significant losses of glutamine are experienced if beet root tissue is dried at 80°C in preparation for analysis.

16462

Plasma Methionine After Oral Administration of *DL-Methionine* in Human Subjects.

HAROLD A. HARPER AND KAHN UYEYAMA. (Introduced by T. L. Althausen.)

From the Department of Biology, University of San Francisco, and the Department of Medicine and Gastrointestinal Clinic, University of California Medical School, San Francisco.

In a recent publication[1] plasma methionine levels after the intravenous administration of 1.5 g of *DL*-methionine were reported for normal human male subjects. In the present communication there is reported a similar study of plasma methionine levels in both male and female normal subjects after oral administration of the amino acid. A few observations on abnormal subjects are included for comparative purposes.

Limited conclusions on the absorption of methionine from the intestine may be drawn

---

[1] Harper, H. A., Kinsell, L. W., and Barton, H. C., *Science*, 1947, **106**, 319.

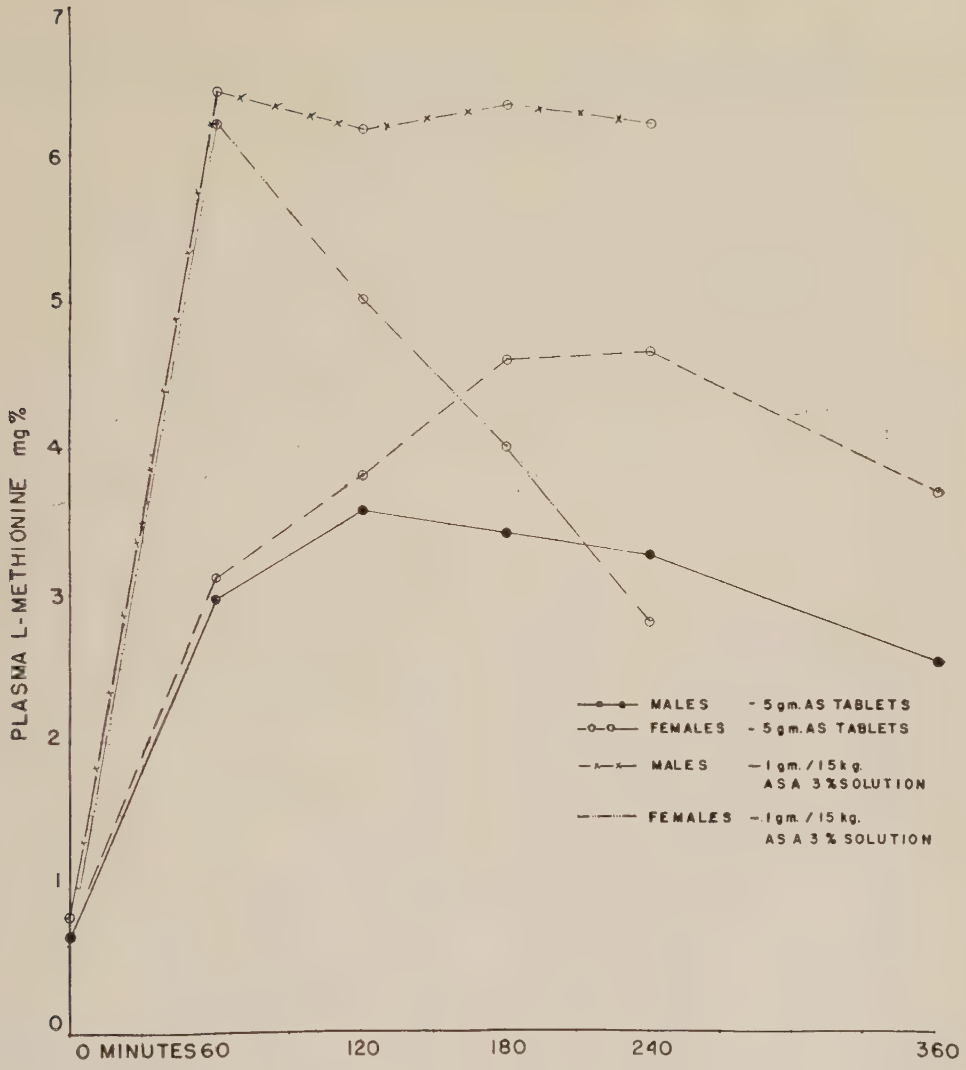


FIG. 1.

Average plasma *L*-methionine levels prior and subsequent to oral administration of *DL*-methionine to normal male and female subjects.

from this study since coincident with the rise in plasma levels by absorption there is a variable rate of uptake by the tissues as well as excretion, particularly of the *D* isomer, both tending to lower the plasma methionine. A similar study of human blood glycine levels after oral administration of this amino acid was recently reported by Gutman and Alexander.[2] Blood tyrosine levels were followed by Bernhart and Schneider[3] in connection with the development of a tyrosine tolerance test. Using the dog as the experimental animal, Hier[4] reported blood values for many individual amino acids after oral administration of a given test amino acid. It is noteworthy that the behavior of methionine was unique in comparison to the other amino acids studied.

Method. Normal subjects for study were

---

[2] Gutman, G. E., and Alexander, B., *J. Biol. Chem.*, 1947, **168**, 527.

[3] Bernhart, F. W., and Schneider, R. W., *Am. J. Med. Sci.*, 1943, **205**, 636.

[4] Hier, S. W., *J. Biol. Chem.*, 1947, **171**, 813.

TABLE I.
Plasma *L*, and Apparent *D*, (*DL-L*) Methionine Levels Prior and Subsequent to Oral Administration of 5 g *DL*-Methionine Tablets.

		Time (min.)											
		0	60		120		180		240		360		
Subject	Sex	*L*	*L*	*D*	*L*	*D*	*L*	*D*	*L*	*D*	*L*	*D*	Remarks
					mg/100 cc								
B.	M	0.56	2.46		3.00		2.84		3.12				Normal
L.	M	1.32	3.04	2.24	3.22	2.98			3.02	3.28	2.10	2.40	”
U.	M	0.50	3.52		4.40				3.88		3.28		”
K.	M	0.28	2.88		3.76				3.20		2.32		”
A.	F	0.56			5.40	5.60	6.30	5.10	5.50	4.70	4.80	5.00	”
Lo.	F	1.04	3.76	2.54	3.36	2.34	4.48	3.42	5.84	0.96	3.76	1.24	”
Le.	F	0.42	3.28		3.88	2.32	4.24	2.46	4.16	2.64	3.40	1.30	”
Q.	F	0.92	3.32	1.88	3.26	2.54	3.30	4.50	2.60	11.60	2.10	17.90	”
Hn.	F	0.30	2.20		3.24		4.88		5.44		4.68		”
C.	M	1.88	4.64	3.86	5.60	3.40	6.48	5.12	6.36	3.24	4.68	4.92	Cirrhosis
Mk.	F	0.20	2.48		3.88	3.22			4.52	2.28	5.24	3.46	Ulcerative colitis
Me.	F	0.32	1.76				3.50		4.88				Massive intestinal resection

7 male and 8 female patients without any demonstrable abnormality which might be expected to influence the results of the test. All were fasted 12 hours before the test dose of methionine was administered and no food was taken during the subsequent period of study. In the first series of experiments a standard dose of 5 g of *DL*-methionine tablets was given, the subject merely swallowing 10, one-half gram tablets followed by a glass of water. In the second series a 3% solution of *DL*-methionine was administered on the basis of body weight in the amount of 1 g per 15 kg. Blood was drawn for a determination of the fasting methionine level and, subsequent to the test dose, at intervals of 60, 120, 180 and 240 minutes with additional samples at 30, 90 and 360 minutes in some cases. Microbiological determinations of plasma *L*-methionine were made employing methods previously described.[1] *Leuconostoc mesenteroides* P-60 was used as test organism for the assay of *L*-methionine and for the *DL* compound, *Lactobacillus fermenti* 36.[5] The difference between the *DL* and *L* values permits estimation of the apparent concentration of the *D* isomer.

Results and Discussion. Fig. 1 depicts the average plasma *L*-methionine levels in normal male and female subjects prior and subsequent to the ingestion of 5 g *DL*-methionine tablets as well as after 1 g/15 kg body weight, *DL*-methionine in a 3% solution. Since relatively few determinations were made at 30 or 90 minutes after the ingestion of the test dose of methionine, these values were eliminated from the curves illustrated in Fig. 1 as well as from the tables to follow. However, in all but one case (Q. female, Table II) plasma values at 60 minutes exceeded those observed at 30 minutes suggesting that absorption was still proceeding at the earlier period. Although the dosages were higher when the tablets were given, the plasma methionine concentration was observed to decline before reaching levels equivalent to those after the solution was administered. In addition in this latter case, there was a tendency for the peak levels to be reached sooner. These observations suggest more prompt and possibly more efficient absorption of the amino acid solution.

The curves illustrating the variation in plasma *L*-methionine after oral administration were somewhat different when males and females were compared. A more rapid rate of disappearance of the administered methionine was observed in females, particularly marked after the solution had been ingested. On the tablet dose, there was a tendency towards the

---

[5] Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *J. Biol. Chem.*, 1946, **163**, 577.

TABLE II.
Plasma *L*, and Apparent *D*, (*DL-L*) Methionine Levels Prior and Subsequent to Oral Administration of a 3% Solution of *DL*-Methionine (1 cc/15 kg actual weight).

			Time (min.)									
			0	60		120		180		240		
Subject	Sex	Dose, g	*L*	*L*	*D*	*L*	*D*	*L*	*D*	*L*	*D*	Remarks
			mg/100 cc									
Hl.	M	5.3	0.50	6.40	12.90	6.10	8.80	6.00	7.50	6.08	4.22	Normal
Z.	M	4.2	0.94	6.88	7.92	6.32	7.18	7.44	2.16	6.48	3.62	ʼʼ
U.	M	5.0	0.40	6.00	5.80	6.24	4.56	5.76	4.44			ʼʼ
Lo.	F	3.7	1.12	5.28	9.22	5.76	4.34	4.24	3.76	2.24	4.24	ʼʼ
W.	F	3.3	0.92	6.08	6.72	4.72	5.38	4.64	2.06	3.68	0.32	ʼʼ
Q.	F	3.6	0.40	7.36	5.34	4.72	3.78	3.28	4.02	2.64	4.56	ʼʼ
K.	F	2.7	0.34	8.64	3.36	5.68	3.12	5.28	2.32	3.60	3.36	Ulcerative colitis
Ha.	F	3.7	1.32	10.40	7.00	9.44	3.96	4.16	7.04	9.28	5.92	Carcinoma of gall bladder
D.	F	2.9	0.48	2.88	2.80	2.72	4.08	1.84	4.44	1.44	4.44	Carcinoma of pancreas
S.	M	3.2	0.44	3.72	4.88	3.84	3.96	3.42	4.18	2.96	1.74	Fever of undetermined origin

attainment of higher plasma levels and at rates slower than those noted in the male on the same dosage. This may be due to the fact that the 5 g dose was high in proportion to size and hence that absorption was more prolonged.

In Hier's experiments[4] very large doses (0.56 g per kg) of methionine were given to the dog and it was noted that, in contrast to any other amino acid studied, blood levels of methionine remained considerably elevated for as long as 24 hours. The doses used in the experiments here reported were proportionately much lower since it was desired to duplicate conditions which would be expected in therapeutic practice. In this dosage range in normal individuals no particular intolerance to methionine has been observed.

Tables I and II detail the plasma *L* and apparent *D* (*DL-L*) methionine levels obtained after the 5 g oral dose or the 1 g/15 kg dose administered orally as a 3% solution. From these data there is no evidence to suggest any variation in rate of absorption of the two isomers. The plasma levels of the apparent *D*-methionine are much more variable than those observed for the *L* isomer. This is certainly due in part to the fact that the rate of removal of the apparent *D* isomer is much more dependent on excretion by the kidney than is the *L* isomer wherein this factor can be neglected at the dosages employed.[6] It is therefore difficult to assign significance to the apparent *D*-methionine plasma levels since, although the rate of absorption seems equal to that of the *L* isomer, the rate of removal from the plasma is influenced not only by metabolic phenomena attendant upon its utilization but also by excretion. Variation in plasma *L*-methionine seems therefore a better indication of the normal physiological phenomena attendant upon absorption and utilization.

Observations on a few abnormal subjects are also presented using both experimental technics. Patient *Me*, female, Table I, had sustained a massive resection of the small intestine, only about 18 inches of the small bowel remaining. However, as evidenced by the increasing plasma levels, absorption was maintained up to the fourth hour suggesting absorption from the colon. That amino acids are absorbed to some extent from the colon was shown by Short and Bywaters in studies of rectal feeding.[7] The male subject with a diagnosis of cirrhosis exhibited a high fasting methionine level and somewhat elevated concentrations after ingestion of the test dose with an initial slow rate of disappearance. This is in accord with other observations in hepatic disease using intravenously administered methionine.[6] The female patient with ulcerative colitis presented a fairly normal curve except for the delayed peak reached at 6 hours.

---

[6] Kinsell, L. W., Harper, H. A., Barton, H. C., Michaels, G. D., and Weiss, H. A., *Science*, 1947, **106**, 589.

[7] Short, A. R., and Bywaters, H. W., *Brit. Med. J.*, 1913, **1**, 1361.

In studies of abnormal subjects using the solution dose (Table II), certain definite deviations from normal were noted. In a case of carcinoma of the gall bladder, extremely high plasma levels of methionine were reached with a rapid fall between 120 and 180 minutes followed by a secondary rise at 240 minutes. In marked contrast, the patient with carcinoma of the pancreas exhibited extremely low plasma levels of methionine with a steady rate of disappearance. A case of ulcerative colitis showed a high initial peak but no other noteworthy abnormality in plasma levels. The last subject with fever of undetermined origin showed a somewhat flat curve suggesting some decrease in intestinal absorption although increased utilization due to an accelerated metabolic rate cannot be excluded.

The abnormal cases presented are not numerous and are intended to serve mainly as comparisons to the normal controls. Further study of this phase of the problem is necessary to establish the validity and significance of the apparent deviations from the normal pattern which have been pointed out.

Summary. 1. The concentration of methionine in the plasma after oral administration of *DL*-methionine has been studied in humans.

2. The results suggest more rapid and more efficient absorption of the amino acid when administered in solution rather than in tablet form.

3. A faster rate of disappearance of the administered methionine from the plasma was noted in the female subjects.

4. Observations on the apparent *D*-methionine levels suggest that the *D* isomer is absorbed at a rate equal to that of the *L* form. However, there is considerable individual variation in the rate of disappearance of the *D* isomer from the plasma.

16463 P

Sodium Oxalate as Anticoagulant.

R. Honorato, C. Rojas, and N. Ivanovic.

From the Laboratorio de Química, Escuela Dental, Universidad de Chile, Santiago, Chile.

It is well known that 0.0025 M solution of sodium oxalate does not delay blood coagulation, even when in stoichiometric concentration with calcium. Three times more sodium oxalate than calcium present in blood is required to prevent blood coagulation (Quick[1]). On the other hand the prothrombin time of oxalated human plasma is prolonged because destruction of the plasmatic cofactor of thromboplastin (P.C.T.) starts a few hours after storage.[3] Quick believes that calcium protects this factor because hemophilic plasma from which calcium has not been removed does not alter its prothrombin time after 48 hours.[2] These experiments do not explain satisfactorily why an excess of sodium oxalate is needed to render blood incoagulable and why oxalated stored plasma destroys by oxidation its cofactor of thromboplastin.[3]

We do not believe that the prothrombin time of oxalated human stored plasma is prolonged because calcium is eliminated. Another possibility must be considered. Sodium oxalate may have more affinity for another substrate than calcium and only when an excess has been added does this last reaction take place. It seems logical to suppose the existence of a

---

[1] Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, 1942, Baltimore, Thomas, Ed.

[2] Quick, A. J., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 249.

[3] Honorato, C. R., *Am. J. Physiol.*, 1947, **150**, 381.

TABLE I.
Prothrombin Time of Fresh and Stored Human Plasma, Decalcified with Amberlite. Results of Oxalate and Citrate Treatment on This Plasma Passed Through Amberlite to Eliminate Calcium.
Prothrombin Time (sec.)

Oxalated fresh plasma	1 day stored	Amberlite plasma	1-day stored Amberlite plasma	1 day stored Amberlite plasma + sodium oxalate	1 day stored Amberlite plasma + sodium citrate
12″	15″	13″	13″	18″	18″

substance in blood which protects the P.C.T. against oxidation. It must be a strong reductor. Its destruction by sodium oxalate would explain the slow but effective alteration of stored plasma.

To test this hypothesis the following experiments were carried out. Human blood withdrawn in silicon syringes (Jaques[5]) was passed through Amberlite IR-100-H (Quick[6]) to eliminate all the calcium without using oxalate. Then 0.1 cc of 0.1 M sodium oxalate or 0.125 M sodium citrate to 0.9 cc of plasma were added. A third sample passed through Amberlite (without addition of any other substance) was stored at 5°C. For prothrombin time determination a modified one-stage method of Quick[4] was used.

The results are shown in Table I.

After 24 hours, no change in the prothrombin time of the sample passed through Amberlite was observed. Thus calcium is not a protective element. On the other hand a very important change in the prothrombin time of plasmas passed through Amberlite, and to which oxalate or citrate were subsequently added, was observed. If no calcium was present, both salts must have altered the plasma by another mechanism than by the elimination of calcium. No difference was observed between oxalate and citrate activity, even when 0.125 M solution for the latter was used (0.125 M citrate preserves plasma better than oxalate 0.1 M, when both are used to withdraw blood samples). We postulate the existence of a protective substance, strong reductor, to preserve P.C.T. from oxidation, which is seriously and specifically altered by sodium oxalate and only subsequently by sodium citrate, 0.125 M, when calcium is present. Without calcium, differences of an affinity of this protective factor disappear.

---

[4] Honorato, C. R., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 41.

[5] Jaques, L., *Can. J. Med. Assn.*, 1946, **55**, 26.

[6] Quick, A. J., *Am. J. Physiol.*, 1947, **148**, 212.

16464

Studies on Polymyxin: An Assay Method for Blood and Urine.*

P. G. STANSLY. (Introduced by M. L. Crossley.)

From the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Conn.

Extension of the agar diffusion method[1] for the assay of polymyxin in fermentation liquors and concentrates has resulted in useful methods for the assay of polymyxin in blood and urine. The method to be described is rapid, simple and relatively accurate and has been useful in studies on the absorption and ex-

---

* Grateful acknowledgment is made to Miss M. E. Schlosser and Mrs. Beverly Gordon for valuable assistance.

[1] Stansly, P. G., and Schlosser, M. E., *J. Bact.*, 1947, **54**, 585.

cretion of polymyxin in experimental animals. It should prove to be equally useful for clinical purposes.

Preparation of seeded plates. Twenty ml of 2% Trypticase-Soy[†] agar are poured into standard petri plates and allowed to solidify. Four ml of 1.2% agar containing 1% "Tween-80"[‡] and 0.5% of a 24-hour, 37°C, Trypticase-Soy broth culture of *Brucella bronchiseptica,* N.R.R.L. B-140, are then spread over the base layer. Prior to use, the plates are placed with lids slightly raised for 45 minutes in a dry 37°C incubator.

Preparation of standard curve for blood. To fresh or citrated normal blood is added an equal volume of 0.2 N HCl. Dry polymyxin hydrochloride is added to an aliquot to give 64 μg per ml.[§] Serial twofold dilutions are made using the remainder of the acid-blood as diluent to give a series of standard solutions ranging from 64 to 0.125 μg per ml. To each of 3 sterile filter paper discs (¼ inch diameter, Schleicher & Schuell No. 740E) in a sterile petri dish is added 0.03 ml of one of the 10 standard solutions by means of a 0.1 ml Kahn pipette. One of these replicate discs is placed in each of 3 seeded plates. The remaining standard solutions are treated similarly. Five discs may conveniently be placed on a single seeded plate. Thus, a total of 6 seeded plates are required for a total of 30 discs, representing the 10 standard solutions in triplicate.

The plates containing the discs are then incubated, in a well-humidified incubator, preferably with lids slightly raised, at 37°C for 16-18 hours and the zones measured in mm. The average zone diameter for each group of 3 replicates is determined and a curve relating zone diameter to log of (concentration × 2) constructed on semi-log paper. A typical blood standard curve is illustrated in Fig. 1.

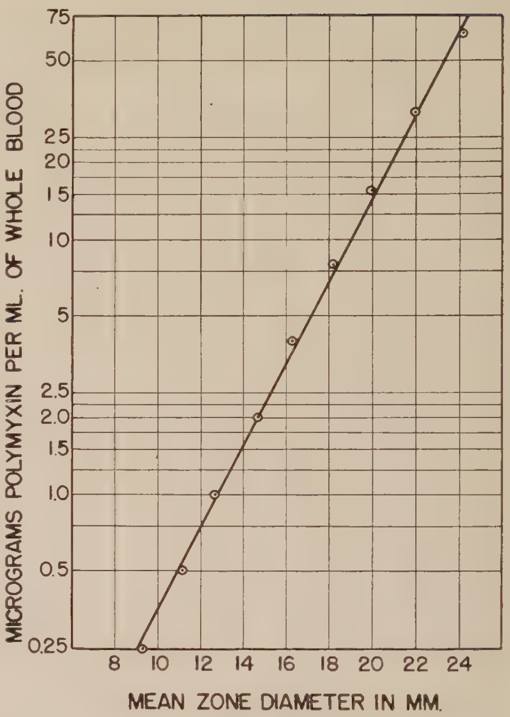


FIG. 1.
Standard curve for assay of polymyxin in blood.

Assay of blood. The manner of collecting blood for assay of polymyxin may be left to the discretion of the individual insofar as the amount of blood removed is concerned and whether the blood is citrated or not. The least amount of blood required is theoretically about 0.02 ml for when diluted with 0.02 ml of 0.2 N HCl, 0.03 ml of solution is available to saturate one disc. If 3 replicates are desired, 0.05 ml of blood is required. Fresh blood may be added directly to the acid or the blood may be citrated and later diluted with the acid. In any case, the saturated discs are treated as described in the preparation of the standard curve. The diameter of the zone is measured in mm and the concentration of polymyxin in whole blood in μg per ml read off the standard curve.

Preparation of standard curve for urine. Serial twofold dilutions of polymyxin from 256 to 0.125 μg per ml are prepared in glycine-hydrochloric acid buffer of pH 2.[‖] The subsequent steps are as described for the preparation of the blood standard curve.

[†] Baltimore Biological Laboratories.

[‡] Polyoxyalkalene derivative of sorbitan monooleate, Atlas Powder Co.

[§] Calculated on the basis of 2000 units per mg for "pure" polymyxin. See *Bull. Johns Hopkins Hosp.*, 1947, **81**, 43.

[‖] One volume of a solution consisting of 7.505 g of glycine and 5.85 g of NaCl per liter of water is added to an equal volume of 0.1 N HCl.

Assay of urine. The sample of urine is diluted with an equal volume of glycine-hydrochloric acid buffer of pH 1.4¶ and subsequently treated as indicated for the assay of blood with the exception that the standard curve for urine is, of course, used to determine the potency in unknown urines.

Discussion. In the assay of fermentation liquors and concentrates for polymyxin,[1] *E. coli* (MacLeod strain) was recommended as the test organism and the two-dose method of determining potency was suggested as more desirable than the standard curve method. *Brucella bronchisepticus,* N.R.R.L. B-140, was recently introduced by Benedict and Stodola[2] as a more desirable organism for the assay of polymyxin. This organism has the advantage of requiring only one temperature for the incubation of the seeded plates and of having a slower rate of growth than *E. coli.* The latter property of *Br. bronchiseptica* permits the standard curve method to be used with less risk of incurring serious error.[1]

Several points are worth considering in connection with the blood assays. In the first place, blood should either be untreated as far as anticoagulants are concerned or citrated. It should not be oxalated. In our experience, oxalated blood frequently results in a viscous solution upon the addition of an equal volume of 0.2 N HCl and is, therefore, difficult to pipette. This does not occur, or only rarely, with either untreated or citrated blood. Under these conditions, solutions of acid blood remain fluid indefinitely at room temperature or in the refrigerator.

In the second place, solutions of polymyxin in 50% acid blood are stable indefinitely in the refrigerator. Therefore, when mixed with acid, the blood samples may be stored until convenient to assay. The series of polymyxin-blood standards may also be stored in the refrigerator indefinitely. It is our practice to prepare a standard curve daily, although this may not be essential if experience indicates that standard curves do not deviate appreciably from day to day.

A third point of interest is that a 50% acid solution of normal blood, prepared as described, does not produce any inhibition zone when applied to a seeded plate and incubated as described. This is true both of animal bloods (mouse and dog) and of human blood (24 miscellaneous samples tested.**) The pH of such solutions is in the neighborhood of 3.75. If stronger acid is used so as to result in a pH of 2, slight but definite inhibition zones are produced which, of course, would complicate the determination of potency in unknown bloods. It is noteworthy that buffer or urine solutions of polymyxin at pH 2 do not exhibit this phenomenon.

Finally, the nature of the assay method precludes the necessity for strict aseptic precautions in the manipulation of the blood for assay and in the various steps of the procedure.

The only point of special interest in connection with the assay of urine is that urine samples are diluted with an equal volume of pH 1.4 buffer, whereas the standard curve is prepared with buffer of pH 2. The reason for this is that the addition of pH 1.4 buffer to urine of our experimental dogs generally results in a pH of about 2, thus bringing it in line with the pH of the standard solutions. The effect of pH of polymyxin solutions on zone diameter has been discussed.[1]

The following examples illustrate the type of data obtained by the methods described.†† A single subcutaneous dose of 1 mg/K of polymyxin hydrochloride in each of 5 mice resulted in a level of 2γ/ml in pooled blood at the end of 15-30 minutes, declining to about 1 γ/ml at the end of one hour; a single intravenous dose of 5 mg/kg in a dog resulted in a blood level of 5 γ/ml of blood at the end of 1½ hours. A total of 90 ml of urine was collected during this time which assayed 220 γ/ml, thus accounting for about 30% of the polymyxin administered. Details of these and other studies will be presented at a later time.

¶ 2.5 volumes of a solution consisting of 9.38 g of glycine and 7.32 g of NaCl per liter of water are added to 7.5 volumes of 0.125 N HCl.

[2] Benedict, R. G., and Stodola, F. H., *J. Bact.,* 1948, **55**, 286.

** Thanks are due Dr. N. G. Chaucer and Miss L. P. Morrison of the Medical Department for their cooperation in supplying samples of human blood for testing.

†† We wish to thank Dr. J. T. Litchfield, Jr., of these Laboratories for permission to quote these results.

Summary. A filter paper disc method for the determination of polymyxin in blood and urine is described. Using ¼-inch diameter discs, 0.25 μg of polymyxin per ml of blood may be determined in triplicate with samples of blood as small as 0.05 ml.

16465

Effect of Digitoxin on Contractility of Ventricular Muscle Strips.

A. M. WEDD AND H. A. BLAIR.

From the Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.

In a summary of his extensive studies on digitalis action, Gold[1] has concluded from the reactions of the papillary muscle of the cat heart that increased systolic force caused by direct action on heart muscle may be taken as the primary mechanism by which digitalis relieves heart failure. Variability of digitalis effect on muscle strips is indicated in numerous reports. Cattell and Gold[2] in carrying out earlier experiments selected as a "therapeutic" dose concentrations which produced augmentation of beat in approximately 50% of trials. Salter and White[3] who used the same procedure found that fresh muscles failed to respond to relatively high concentrations of ouabain, and in a later paper[4] they described alteration of the Ringer solution in addition to prolonged stimulation, so that the muscle might be more readily "fatigued." In their study of digoxin on strips of turtle ventricle, Wedd, Blair, and Dwyer[5] rarely observed pronounced or sustained increase in amplitude of contraction and argued that the constant shortening of systole, electrical and mechanical, might be a factor in reducing contraction height. It is apparent from such studies that augmentation of contractility by digitalis is not due to primary stimulation of that function, such as occurs with epinephrine or veratrine. Further observations indicate that to be enhanced contractility must have first been impaired and experiments to be cited suggest that such effect will occur only after some alteration of muscle metabolism. The present report deals with the effect of digitoxin on strips of turtle ventricle when contractility has been diminished by various physical and chemical means.

Method. Details of the method used in this laboratory to study drug effects on heart muscle have been described.[5] Ventricular strips suspended in a bath are rhythmically stimulated by condenser discharges and contraction recorded on a drum. The Ringer solution was phosphate buffered and had a pH of 7.2; no carbonate or sugar was present. The usual driving rate was 13.5 per minute. Tension was adjusted to record maximum contraction; it was altered if necessary during the control period, but not after the drug was added. It made no difference whether digitoxin was added to the original bath or a fresh one containing the drug was substituted. A concentration of 1:2 million was found generally satisfactory and was used in all experiments.* For each group in which con-

---

[1] Gold, H., *J. Am. Med. Assn.*, 1946, **132**, 547.

[2] Cattell, McK., and Gold, H., *J. Pharm. Exp. Therap.*, 1941, **71**, 114.

[3] Salter, W. T., and White, W. F., *Fed. Proc.*, 1946, **5**, 200.

[4] White, W. F., and Salter, W. T., *J. Pharm. Exp. Therap.*, 1946, **88**, 1.

[5] Wedd, A. M., Blair, H. A., and Dwyer, G. K., *J. Pharm. E Therap.*, 1941, **72**, 394.

* The digitoxin used in these experiments was Digitalline Nativelle, generously supplied by the Varick Pharmacal Co.

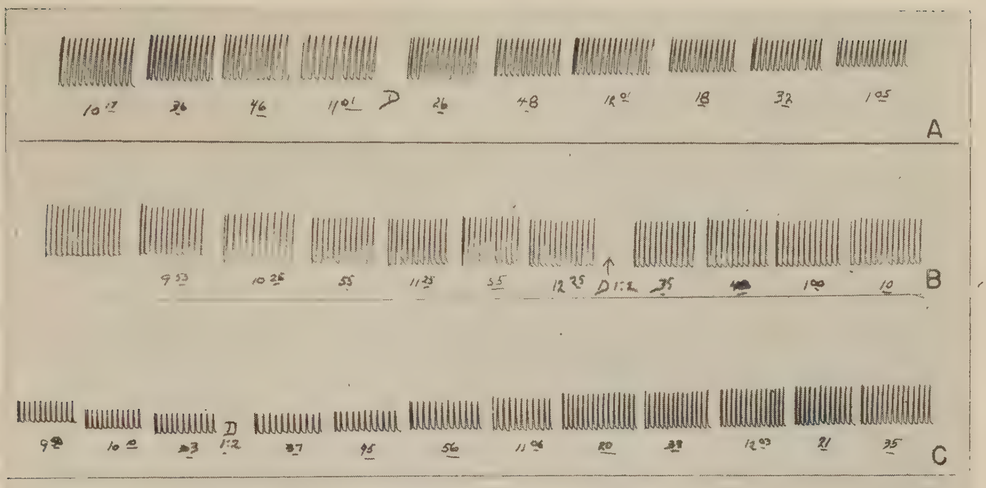


Fig. 1.
Illustrating the types of response of untreated muscle strips to digitoxin; concentration 1:2 mil in each instance. Lower figures indicate time.

tractility was artificially depressed at least 4 experiments were performed. When a positive inotropic response was obtained increase in contraction height ranged from 15 to 50%. The maximal effect restored beat size to its original level but never raised it beyond that. Relationship of electrical to mechanical systole which was also studied in these experiments will not be considered in detail, but it may be stated here that most of the agents used to depress contractility simultaneously shortened electrical systole, measured by the Q-T interval of the electrogram.

Untreated strips. Three types of response to digitoxin have been observed for untreated ventricular strips tested at varying times up to 96 hours after removal from the animal. A concentration of 1:3 million usually produced no effect on the beat. The standard test concentration, 1:2 million, in some strips caused a decrease in beat and considerable diastolic shortening, illustrated in Fig. 1, A. While there is not necessarily any relation between muscle contraction and recovery, it is possible that under certain conditions shortening the duration of systole may itself lead to a decrease in beat size and then such beat decrease is not an expression of toxicity. Shortening of diastolic length is perhaps more often seen in the turtle than in mammalian strips; the presence of varying amounts of smooth muscle in the turtle ventricle may be a factor. For other strips the standard concentration was without appreciable effect, Fig. 1, B. Certain turtles presented hearts whose muscle appeared to be in poor condition and the maximal beat was feeble for the size of the strip. It was in such strips or in muscles that showed progressive decline during the control period that contraction was significantly augmented by digitoxin, Fig. 1, C. There were also instances in which a poor beat was unchanged or continued to decline after the drug. Transient improvement of beat followed by decline and shortening such as Gold described and related to toxic dosage was not seen with the concentration used in these experiments.

Fatigue. It was found difficult to produce a decline in beat in fresh strips by stimulation alone. However, this was accomplished in a few by driving at double the usual rate. Digitoxin then caused further decline and some shortening or there was no appreciable change, results similar to those already described for strips driven at the slow rate. But in older strips a slight beat increase or at least arrest of decline followed digitoxin. In one experiment the strip which had been out of the animal for 72 hours showed a decrease of only 1.5 mm after driving for 3 hours; the following day, after 16 hours, there

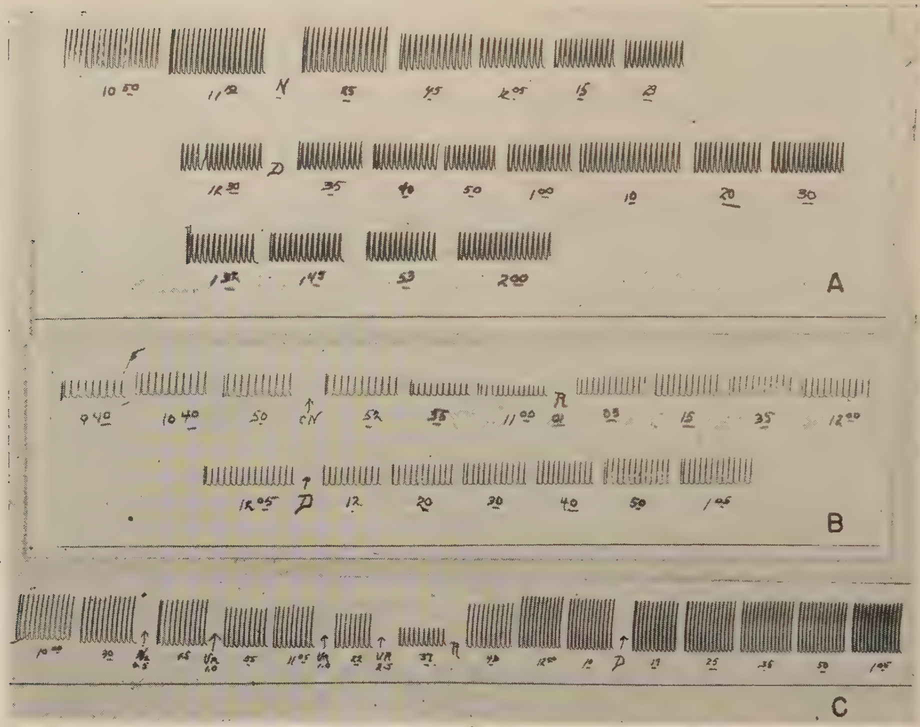


FIG. 2.

A: Depression of beat by nitrogen and improvement following digitoxin added at D. B: Depression by sodium cyanide; recovery in fresh Ringer solution, R; further increase after digitoxin, D. C: Depression by urethane; complete recovery in fresh Ringer solution, R; no further change after digitoxin.

was a considerable decrease in beat, followed by a slight increase after digitoxin. In another strip tested after 72 hours the beat decreased 40% in 45 minutes; there was a gradual increase after digitoxin so that at the end of 2 hours the beat size was up 25%. These experiments suggest that decline in beat due to rapid stimulation is not necessarily related to metabolic changes which will be favorably influenced by digitoxin, and that for a positive inotropic response "fatigue" alone is insufficient.

Cold. Cooling the bath containing the muscle from room temperature, 21-22°C, to 9° reduced the size of the beat by half and digitoxin caused no increase. Further lowering of temperature resulted in alternation and then alternate response.

Increased Acidity. By addition of HCl the pH of the bath was decreased to 6.5 and to 6.0. Such changes caused a decrease in contraction height which digitoxin could in part offset. The drug prevented further decline or increased the size of the beat, though it never restored it to the original height.

Other Ionic Changes. Ringer solution which contained half the normal amount of calcium caused little or no change in beat. In a calcium free solution the beat decreased greatly and that effect was not counteracted by digitoxin. The addition of a very small amount of calcium promptly restored the beat. In a bath of isotonic sodium chloride, alone or with phosphate buffer, the beat was greatly reduced and not improved by digitoxin. Excess potassium caused the beat to decline, and again digitoxin did not increase it. Increasing potassium to 7 times the normal also caused alternation and alternate response. In one experiment the bath was first changed to a chloride-phosphate solution and the beat fell to half; it further declined after digitoxin and then still further on adding the normal quantity of potassium; with the addition of the normal amount of calcium the beat promptly increased, almost to its original level.

Nitrogen. The bubbling of pure nitrogen through the bath instead of air caused a prompt and progressive decline in beat. In one instance only was digitoxin able to offset the depressant effect while that gas was still present, Fig. 2, A. If after an hour in nitrogen the bath was again aerated recovery of beat occurred. Often recovery was incomplete, and then digitoxin regularly caused further increase in beat size.

Chemical Depressants. The action of sodium cyanide, sodium azide, hydroxylamine and urethane was similar. Each in suitable concentration depressed contractility. It was necessary to permit recovery in fresh Ringer solution; otherwise the addition of digitoxin caused further decline and shortening. When recovery was partial or not sustained digitoxin increased the beat; the response to digitoxin after recovery from depression by sodium cyanide is illustrated in Fig. 2, B. Urethane was a potent depressant and following its removal recovery was often complete; then digitoxin caused no further increase in the beat, Fig. 2, C. Concentrations of iodoacetate and of sodium fluoride sufficient to cause an appreciable decrease in beat likewise tended to produce much diastolic shortening. There was little tendency to recover from the shortening and the change to fresh Ringer solution was often followed by additional shortening; that reaction was further increased by the addition of digitoxin. None of the foregoing substances, which were tried for their property of interfering with enzyme systems, gave reactions which were sufficiently specific to indicate which phase of metabolic activity might have been disturbed. Even cyanide is thought to have an effect on contractility independent of its interference with oxygen utilization. The results of the group in general were similar to those obtained with nitrogen gas. The effect of quinidine was to depress contraction in strips driven at the rapid rate; digitoxin did not improve the beat in the presence of the former drug; in fresh Ringer solution recovery was incomplete and then digitoxin increased the beat.

Comparison of certain glycosides. The effect upon mechanical response and on electrical systole was studied for ouabain, digitoxin, digoxin and lanatoside C. While these substances produced practically equivalent shortening of the Q-T interval of the electrogram,[6] their influence on the mechanical response appeared unequal. Because of its relatively greater viability and perhaps variability of response the turtle ventricle is probably not so suitable for the study of comparative contractility effects as mammalian heart. However, certain results are in agreement with reported findings for these drugs. Of the 4 substances ouabain was most potent. A concentration of 1:10 mil usually produced no effect on beat size; in untreated strips, 1:7.5 mil and greater concentrations caused decline in beat and shortening. In strips that had been depressed by chemicals, 1:5 mil increased the beat without significant shortening; higher concentrations first increased and then depressed contraction. Digitoxin and digoxin in concentration of 1:2 mil appeared approximately equivalent to 1:5 mil ouabain, and the equivalent concentration of lanatoside C was 1:3 mil. While these experiments cannot be said to be conclusive the indicated concentration ratios are 1:1.65:2.5 for ouabain, lanatoside C, and digitoxin or digoxin. When compared on the basis of molar concentration the corresponding ratios are 1:1:1.9. Although ouabain appeared of considerably greater potency, in a series of trials on chemically depressed muscles effective concentrations did not act more promptly or cause greater increases in contraction height than digitoxin.

Summary. Study of the action of cardiac glycosides on strips of turtle ventricle indicates that the positive inotropic effect produced by those drugs does not result from primary stimulation of contractility but that it will occur only after there has been some suitable change in muscle metabolism. Beat depression caused by rapid stimulation, cold, calcium want, or excess potassium was not improved by digitoxin. Reduced contraction height due to increased acidity was partly offset by digitoxin. When contractility was depressed by various chemicals digitoxin usually increased the beat provided spontaneous recovery had been incomplete.

---

[6] Wedd, A. M., and Blair, H. A., *J. Pharm. Exp. Therap.*, 1947, **90**, 211.

Ouabain produced effects comparable to digitoxin with approximately half the concentration. It did not act more promptly or cause relatively greater increases in beat.

16466 P

pH Stability, Response to Antibiotics and Factors Influencing Egg-Culture of Mumps Virus.*

M. L. WEIL, DOROTHY BEARD, AND J. W. BEARD.

From the Department of Surgery and the Department of Pediatrics, Duke University School of Medicine, Durham, N.C.

Like the agents of influenza the mumps virus grows in embryonated eggs,[1-4] reaching high concentrations in the chorio-allantoic fluid. Studies have been undertaken for the purification and examination of the agent by physical and chemical methods.

Preliminary to the development of purification procedures, studies have been made on (a) the conditions influencing the concentration of the virus in the chorio-allantoic fluid; (b) the effects of antibiotics on the virus and on (c) the pH stability of the virus. The results of this work are briefly given here, and the purification and characters of the virus are described in an accompanying report.[5]

Material and Methods. The virus was the Enders chick embryo adapted strain,[2] obtained through Dr. Lalla Iverson. The methods of inoculating the virus into the chorio-allantoic sac and harvesting the chorio-allantoic fluid were similar to those used in studies of influenza virus.[6] Virus infectivity was titrated in 7-day-old embryos. Hemagglutinative capacity[2,7] was measured by the method of Hirst[8] as used with the influenza virus.[9]

To avoid bacterial growth the use of antibiotics seemed desirable. The effects on the virus in chorio-allantoic fluid of penicillin (500 units per cc) and streptomycin (100 μg per cc) separately, and in combination in the same respective concentrations, were studied in *in vitro* experiments. The mixtures were stored at 4°C and titrated after 20 minutes, 7, 14 and 28 days.

The pH stability of virus infectivity was examined in a composite buffer[10] of borate, phosphate and phthalate (0.04 M total concentration) containing penicillin, 500 units per cc and streptomycin, 100 μg per cc. To 16 cc aliquots of the buffer adjusted to pH values at intervals of 1 pH unit over the range of 2.0 to 11.05, 4 cc of virus-containing chorio-allantoic fluid were added. The mixtures were kept at 4°C, and infectivity measurements were made at intervals of 1, 8, 15 and 29 days.

---

* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N.Y.

[1] Habel, K., *Pub. Health Rep.*, 1945, **60**, 201.

[2] Levens, J. D., and Enders, J. F., *Science*, 1945, **102**, 117.

[3] Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 15.

[4] Beveridge, W. I. B., and Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1947, **25**, 337.

[5] Weil, M. L., Beard, D., Sharp, D. G., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 309.

[6] Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

[7] Beveridge, W. I. B., and Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 305.

[8] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[9] McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 305.

[10] Best, R. J., and Samuel, G., *Ann. App. Biol.*, 1936, **23**, 509.

Virus growth was studied in embryos of 7, 8 and 9 days incubation at 37.5°C before inoculation; hemagglutination was measured daily after the 4th day of incubation at 35°C until a total incubation of 15 days. Infectivity measurements were made in parallel with the fluid from the eggs 7 days old at inoculation. The influence of concentration of virus in the inoculum was examined with dilutions of chorio-allantoic fluid of 10^{-0}, 10^{-1}, 10^{-2} and 10^{-3}.

Results. There was no evidence of inactivation *in vitro* of the virus by the antibiotics. Penicillin[3] and streptomycin in concentrations of 500 units and 100 μg per cc introduced with the inoculums (0.05 cc) did not hinder virus growth in the egg.

The greatest increase in virus hemagglutinative capacity occurred in 7-day eggs with the hemagglutinative units per cc and total hemagglutinative units increasing to the 8th day after inoculation. Infectivity in 7-day embryos reached a maximum at the 4th to 6th days after inoculation and diminished thereafter. Infectious titers per cc of pooled fluids have varied from $10^{-8.5}$ to $10^{-9.5}$ and the hemagglutination units (2 +) from 45 to 128 per cc. Titers of individual eggs vary widely, from 22 to 360. The concentration of virus in the inoculum was not an influencing factor.

The mumps virus has been very unstable under any conditions thus far studied. Titers in chorio-allantoic fluid at 4°C diminished as much as from $10^{9.0}$ to $10^{7.4}$ infectious units per cc within 2 weeks. The range of optimum pH stability was from about pH 5.65 to 7.9. Maximum stability appeared to be at pH 6.5 to 7.0. Inactivation was very rapid below pH 4 and above pH 9.5. Little inactivation occurred within 8 days in the range of pH 5.65 to 7.9; after 29 days the titers in this range were about 2 10-fold dilutions lower than the initial level.

Summary. Neither penicillin nor streptomycin, separately or combined, caused demonstrable inhibition of the mumps virus *in vitro* or *in vivo*. Maximum virus infectivity occurred in 7-day-old embryos 4-6 days after inoculation; hemagglutinative capacity reached a maximum in like embryos after 8 days of incubation. The concentration of virus containing chorio-allantoic fluid used as inoculum had no apparent effect on growth of the virus in eggs. The range of optimum pH stability was 5.65 to 7.9, but the virus was relatively unstable under any conditions.

16467 P

Purification and Sedimentation and Electron Micrographic Characters of the Mumps Virus.*

M. L. WEIL, DOROTHY BEARD, D. G. SHARP, AND J. W. BEARD.

From the Department of Surgery and the Department of Pediatrics, Duke University School of Medicine, Durham, N.C.

In an accompanying paper,[1] there are described the results of experiments on the growth and stability of mumps virus carried out preliminary to studies on the purification of the virus. The purification and the sedimentation and electron micrographic characters of the agent are described here.

Material and Methods. The general methods employed in the work have already been described.[1] The crude chorio-allantoic fluid harvested 5 days after inoculation of 7-day-old chick embryos, and held at 4°C for 1-4 weeks, was spun in an angle centrifuge at 1,700 g for 15 minutes. The supernate was then filtered through No. 1 Whatman filter paper and spun in the horizontal head at 3,000 g for 1 hour. This supernate was centrifuged at 10,000 g for 1 hour to sediment the virus, and the pellets were resuspended in ½ the initial volume in half strength Ringer solution containing glycine in 0.01 M concentration. This suspension was then spun at 10,000 g for 5 minutes, and the resulting supernate centrifuged again at 10,000 g for 45 minutes. The second cycle pellets were resuspended at 56 × concentration in Ringer-glycine solution. The suspension was then centrifuged in the angle head at 625 g for 10 minutes. The supernate contained the concentrated and purified virus. All manipulations were carried out in the cold. Sedimentation studies were made with the air-driven analytical ultracentrifuge using the absorption method of Svedberg and also the Lamm scale procedure.[2] The electron microscope was the RCA type B instrument.[3]

---

* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N.Y.

[1] Weil, M. L., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 308.

Results. The average yield of chorio-allantoic fluid has been 5-6 cc per egg and the pH of pooled fluids about 8. Filtration through filter paper seems to remove mucoid and other extraneous material. The sedimented virus is very difficult to redisperse. Aggregation occurs readily in 0.9% NaCl; 0.005 to 0.05 M phosphate buffer; and in Ringer solution. The best results were obtained with the dilute Ringer-glycine solution of pH 7.4. The pellets of the first sedimentation contained a central area of yellowish material which redispersed with the virus. Some of this was eliminated in the brief centrifugation at 10,000 g. The pellets of the second sedimentation were nearly clear, containing little opaque material.

The final yield of hemagglutinative factor was from 31-60% of the starting material; the virus infectivity recovered was 5-13%. The chief losses in infectivity appeared to be related to inactivation of the virus in the centrifugal procedures. The 2+ hemagglutinative unit was $10^{-7.19}$ g of nitrogen and the infectious unit for chick embryo was $10^{-13.76}$ g of nitrogen. The yields were from 0.25 to 0.45 mg of virus nitrogen per 100 cc of chorio-allantoic fluid.

FIG. 1.
Sedimentation diagram of mumps virus. The concentration was 143 γ of virus nitrogen per cc. The interval between exposures was 3 minutes and the centrifugal field 4,100 g.

Fig. 1 is the sedimentation diagram of a preparation containing 143 γ of virus nitrogen per cc. The broad spread in the boundary indicates a wide variation in particle size which is confirmed by the electron micrographs. A similar spread in boundary was seen in the Lamm scale diagrams. Measurements of successive peaks gave an average sedimentation rate of $S_{20}° = 1330 \times 10^{-13}$ for the virus.

Much difficulty has been experienced in obtaining electron micrographs. The images of the agent in the electron microscope have been indefinite and are of irregular shape. Micrographs of purified virus treated for 31 days with 0.1% formalin are shown in Fig. 2 and 3. The images under these conditions appear approximately circular and exceedingly variable in size ranging from 106 to 282 mμ with an average of 190 mμ and a standard deviation of 42 mμ. There is only a suggestion of internal material of high absorbing power for electrons. In the shadowed[4] micrograph there is a great similarity between individual particles although the same variation in image size is seen. The particles are flattened and show a roughened, irregular surface contour somewhat similar to the elementary bodies of vaccinia.[5] The shape of the formolized virus

---

[2] Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge*, Oxford, 1940.

[3] Hillier, J., and Vance, A. W., *Proc. Inst. Radio Engrs.*, 1941, **29**, 267.

[4] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

[5] Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 295.

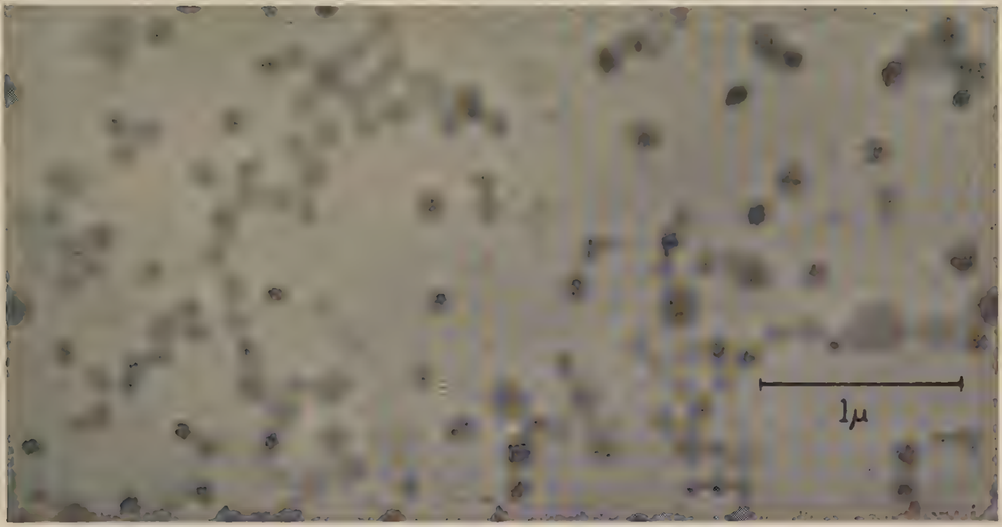


FIG. 2.
Electron micrograph of a mumps virus concentrate after treatment with 0.1% formalin for 31 days. Magnification = 27,320 ×.

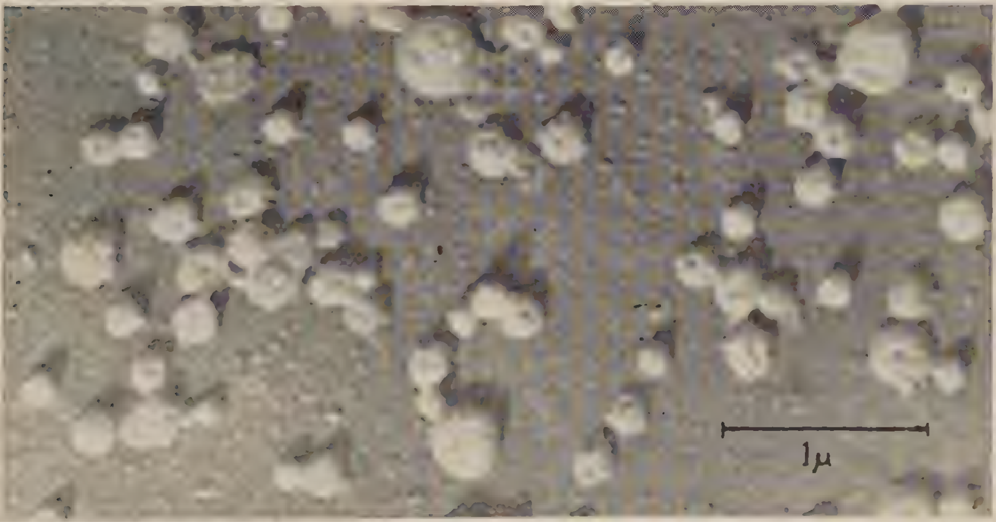


FIG. 3.
The virus preparation of Fig. 2 shadowed with 19 A° of chromium at an angle whose tangent was 2/8.5. Magnification = 28,800 ×.

was inferred to be spherical. This preparation showed relatively very few of the small particles the size of the normal component of chorio-allantoic fluid.

Summary. The mumps virus has been purified by centrifugation of virus-infected chorio-allantoic fluid. Sedimentation diagrams indicate wide variation in particle size and a sedimentation rate of about 1330×10^{-13}. The electron micrographs corroborate the centrifugal evidence of variation in particle size. The shape of the formolized virus was judged to be spherical and flattened electron micrograph images have a diameter of 190 ± 42 mμ.

16468 P

Inhibition by Egg-White of Hemagglutination by Swine Influenza Virus.*

Frank Lanni and J. W. Beard.

From the Department of Surgery, Duke University School of Medicine, Durham, N.C.

Shortly after the discovery of influenza virus hemagglutination[1,2] it was reported[3] that normal serum possessed the capacity to inhibit this phenomenon in low dilution. Subsequent investigation showed that a variety of other materials possessed a similar capacity.[4,5]

Recently, Francis[6] demonstrated that the inhibition by normal serum was enhanced if heated virus preparations were used in the titration of inhibitor. The present report is concerned with the preliminary description of an analogous, but much more striking, inhibition phenomenon with egg-white.

Experimental. Preliminary experiments have been carried out with a purified preparation of egg-adapted swine influenza virus[†7] and a derivative obtained by heating a buffered-saline suspension of the virus for 30 minutes at 53°C, at a concentration of 1.0 γ N per ml. Fresh egg-white (EW) was strained through gauze and diluted serially with buffered saline for the tests. Typical inhibition titers, determined by the standard hemagglutinative method of this laboratory[7] and expressed as the reciprocal of the final dilution of EW required to neutralize two to three hemagglutinating units of virus, averaged 170,000 with heated virus; and, with one egg-white which was investigated in detail, the enhancement over the inhibition titer for unheated virus was 750- to 1,000-fold. With the same virus preparations, a pool of normal rabbit serum gave an inhibition titer of 760 with heated virus and 120 with unheated virus, the Francis enhancement thus being only 6-fold.

A striking susceptibility to inhibition by EW was shown also by an aged formolized swine influenza vaccine,[7] with which titers of about 54,000 were obtained, but not by a freshly prepared vaccine, which exhibited a behavior identical, in this respect, with that of unheated virus. The ratio of about 1 to 3 for the inhibition of aged vaccine and heated virus was found with each of 13 individual whites, and we may accordingly suppose that these 2 virus preparations presented the same differences to each of the egg-whites tested and that the inhibitory property is ascribable to a characteristic ingredient of EW.

The EW inhibitor is almost completely destroyed by treatment with dilute periodate (Table I), which has been shown to destroy the red cell receptor[5,8,9] and the normal serum inhibitor.[4,5,9] In view of the current belief[4,5] that both the red cell receptor and the normal serum inhibitor are mucoproteins, it is interesting to note that when EW, either undiluted or diluted 5-fold with buffered saline, is treated with an equal volume of a saturated aqueous solution of potassium periodate, there is formed a considerable mucin-like precipitate, consisting possibly of ovomucin.[10,11]

We have further observed that when undiluted or 20% EW is overlayered with a con-

---

* This work was aided by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N.Y.

[1] Hirst, G. K., *Science*, 1941, **94**, 22.

[2] McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, **32**, 530.

[3] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[4] Burnet, F. M., *Lancet*, 1948, **254**, 7.

[5] Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301.

[6] Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

[7] McLean, I. W., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

[†] Strain 15, originally obtained from Dr. R. E. Shope.

[8] The Rockefeller Foundation, International Health Division, Annual Report, New York, 1945, 50.

[9] Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160**, 404.

[10] Hektoen, L., and Cole, A. G., *J. Infect. Dis.*, 1928, **42**, 1.

[11] Young, E. G., *J. Biol. Chem.*, 1937, **120**, 1.

TABLE I.
Effect of Dilute Potassium Periodate on the Capacity of Egg-white (EW) to Inhibit Hemagglutination by Heated and Unheated Swine Influenza Virus.

EW sample	Treatment*	Inhibition titer†	
		Unheated virus	Virus heated 30 min. at 53°C
1	1.0 ml 20% EW (in buffered saline, pH 7.4) + 2.0 ml buffered saline	60	100,000
2‡	1.0 ml 20% EW + 1.0 ml sat. KIO_4 (<0.05 M); after 2 hrs, add 1.0 ml 10% glucose§	<60	60
3	1.0 ml sat. KIO_4 + 1.0 ml 10% glucose; after 2 hrs, mix with 1.0 ml 20% EW	60	33,000
4‡	1.0 ml 20% EW + 1.0 ml sat. KIO_4; mix and add 1.0 ml 10% glucose immediately	<60	450
5	1.0 ml 20% EW + 1.0 ml H_2O; mix and add 1.0 ml 10% glucose immediately	60	100,000
6‡	1.0 ml 20% EW + 1.0 ml 10% glucose; mix and add 1.0 ml sat. KIO_4 immediately	<60	3,200

* Carried out at room temperature (29°C).

† Determined with 4 two-plus units of virus. Titers expressed as the dilution of whole egg-white.

‡ Considerable mucin-like precipitate formed immediately on the addition of periodate and was removed by centrifuging at the end of the two-hour reaction period. Judged visually, the greatest precipitate formed in sample 2, next in sample 4, and least in sample 6. The results suggest that part of the decrease in inhibition titer may be attributable to the formation of this precipitate.

§ Glucose was used to inactivate the residual periodate.

centrated suspension (about 10 γ N per ml) of unheated virus or aged vaccine, reaction with virus is directly manifested by the formation of a precipitate at the interface. Furthermore, this property of EW, like its inhibitory property, is destroyed by treatment with periodate. In addition, we have found that the similar capacity of normal rabbit serum to precipitate with concentrated virus preparations[12,13] is likewise destroyed by treatment of the serum with periodate.

Discussion. The present total of evidence, in particular, the direct observation of reaction between EW and virus, the parallelism in the effect of periodate on the precipitation and inhibition reactions, and the dependence of inhibitor activity on the state of the virus, suggests that the capacity of EW to inhibit virus hemagglutination is attributable to a component which combines with the virus.

Summary. Egg-white (EW) has been found approximately 200 times as effective as normal rabbit serum in inhibiting hemagglutination by heated swine influenza virus, giving titers of about 170,000. An aged formolized vaccine was also highly susceptible to inhibition by EW, but unheated virus and a fresh vaccine required about 750 to 1,000 times as much EW for similar inhibition. In addition, EW gave an interfacial precipitation reaction with concentrated virus suspensions, and both the precipitating and inhibiting capacities were destroyed by treatment with dilute periodate.

---

[12] Knight, C. A., *J. Exp. Med.*, 1944, **80**, 83.

[13] Knight, C. A., *J. Exp. Med.*, 1946, **83**, 281.

16469

Chemotherapy of Joint Involvement in Mice Produced by *Streptobacillus moniliformis.*

Jennie S. Levey and Stanley Levey. (Introduced by Arthur H. Smith.)

From the Wayne County General Hospital, Eloise, and the Department of Physiological Chemistry, Wayne University College of Medicine, Detroit.

Both in rat bite fever and in Haverhill fever where *Streptobacillus moniliformis* is the etiological agent, patients may experience joint involvement among other symptoms.[1-4] This microorganism has also been recovered from mice which have developed spontaneous arthritis.[1,5] Most of the chemotherapeutic studies involving *Streptobacillus moniliformis* have been concerned with the protective effect of the various agents when administered prior to or at the time of inoculation of the test animals. In the present study the animals were inoculated and allowed to develop swollen joints before the test agents were administered. In this manner it was possible to evaluate the therapeutic effect of various substances. The drugs tested were chosen because they possess chemotherapeutic value in various other infectious diseases and in addition possess different chemical structures.

Methods. The Manning strain of *Streptobacillus moniliformis* used in this work was supplied by Dr. Carl E. Duffy, Wayne University College of Medicine. It was isolated from the blood of a man who had developed painful and swollen joints following a fall. There was no history of a rat bite. The typical "fluff-ball" growth of this microorganism appeared readily in either tryptose phosphate or freshly prepared beef heart infusion broth[6] supplemented with Seitz-filtered bovine serum to a final concentration of 20%.

Broth cultures of low passages of the Manning strain were stored in small amounts in solid carbon dioxide. When needed, they were thawed quickly in a water bath of 37°C and transferred to flasks of sterile 20% bovine serum broth. Two to five-tenths of a ml of either a 24- or 48-hour broth culture was administered intravenously to mice weighing approximately 20 g. Subcultures up to the seventh passage were used. The microorganism proved to be so virulent for these mice that extremely swollen joints appeared as early as 2 days after the inoculation. If the individual subculture caused the death of most of the animals 48 hours after inoculation, the smaller dose level (0.2 ml) was used. Any of the mice which failed to show swollen joints were discarded.

The substances tested were: penicillin G, streptomycin, Myochrysine (gold sodium thiomalate), Solgonal B* (gold thioglucose in oil), sulfathiazole, p-aminosalicylic acid,† p-aminobenzoic acid, promizole† (2-amino-5-sulfanilylthiazole), promin† (disodium salt of P, P′ diamino-diphenylsulfone-N, N′ diglucose sulfonate) and methylene blue. Therapy was begun 3 days after the appearance of one or more swollen joints. Since the mode of treatment varied with the individual drug, the dosage will be described in the discussion of the specific agent. The evaluation of the therapy depended on the complete disappearance grossly of all joint involvement. Reduc-

---

[1] Brown, T. McP., and Nunemaker, J. C., *Bull. Johns Hopkins Hosp.*, 1942, **70**, 201.

[2] Place, E. H., Sutton, L. E., and Willner, O., *Boston M. and S. J.*, 1926, **194**, 285.

[3] Place, E. H., and Sutton, L. E., *Arch. Int. Med.*, 1934, **54**, 659.

[4] Parker, F., Jr., and Hudson, N. P., *Am. J. Path.*, 1926, **2**, 357.

[5] Williams, S., *M. J. Australia*, 1941, **1**, 357.

[6] Zinsser, H., and Bayne-Jones, S., *A Textbook of Bacteriology*, D. Appleton-Century Co., New York, 1935, p. 1056.

* This compound was supplied through the courtesy of the Schering Laboratory, Bloomingdale, N.J.

† These compounds were supplied through the courtesy of Dr. L. L. Bambas of Parke, Davis and Company, Detroit, Mich.

TABLE I.
Effect of Various Agents upon Joint Involvement Produced by *Streptobacillus moniliformis.*

Group	Drug	Course of therapy	No. of animals tested	No. of animals cured	% cured
1	Control		63	1	1.6
2	Penicillin G	10,000 units I.P., 3 × daily, 4 days	32	12	37.5
		1,000 ’’ ’’ ’’ ’’ ’’ 6 ’’	16	11	69.0
3	Streptomycin	5,000 ’’ ’’ ’’ ’’ ’’ 3 ’’	7	6	85.0
		2,500 ’’ ’’ ’’ ’’ ’’ 3 ’’	17	13	76.0
4	Myochrysine	1.25 mg I.V. every other day, 10 doses	17	0	0
		5 mg I.P. single dose	5	0	0
		4.0 mg I.V. every other day, 10 doses	8	0	0
5	Solgonal-B in oil	10 mg I.M. single dose	7	0	0
6	Sulfathiazole	1 % in diet, 38 days	11	0	0
7	*p*-Aminosalicylic acid	.5% ’’ ’’ 9 ’’	13	0	0
8	*p*-Aminobenzoic acid	.4% ’’ ’’ 21 ’’	10	0	0
9	Promizole	.5% ’’ ’’ 5 ’’	13	1 (?)	0
10	Methylene blue	.2% ’’ ’’ 20 ’’	5	0	0
11	Promin	.5% ’’ ’’ 21 ’’	22	0	0

tion of the number of limbs involved without complete remission was not considered a cure. The mice were observed for a period of 3 months; if any of the mice died during the first 3 days of treatment their records were discarded. For each series of tests a similar number of controls was employed.

Results. Of the 63 mice used as controls, complete remission of the swollen joints occurred in only one case. There was usually an increase in the number of joints involved for a week following the inoculation of the organisms, after which the number remained constant.

When 10,000 units of penicillin G were injected intraperitoneally 3 times a day for 4 days, 12 of the 32 mice (37.5%) made apparent gross recovery. However, 69% of another group of mice which received a smaller dose of penicillin G 3 times a day for 6 days were cured (Table I).

Streptomycin produced even more striking results. There was an 85% recovery in 7 mice when they were given 5,000 units intraperitoneally 3 times a day for 3 days. Of the 17 mice which received 2,500 units 3 times a day for 3 days, 13 lost all signs of previous swelling of their limbs (76%). Because of the unavailability of streptomycin at the time this study was initiated, more test animals could not be employed.

The 2 gold compounds which were studied at various dose levels did not have any effect on the joint involvement in the mice.

All the other substances used were added in various amounts to ground dog chow (Purina) which comprised the diet of the mice. In a group of mice which had been fed 1% sulfathiazole for 38 days none of the animals showed recovery. The level of sulfathiazole used was that suggested by Barlow and Hamburger.[7]

Another series of mice received 0.5% p-aminosalicylic acid in their diet but since so many of the mice died the supplementation was discontinued after 9 days. Again there was no improvement in the mice. After a few days on a diet containing 0.5% promizole in ground dog food, the tails and feet of the mice in Group 9 (Table I) turned blue. The diet was discontinued for a week and then resumed. Not only did the same results recur, but there was such a high percentage of fatality that the mice were taken off the diet. Bambas also observed that both promizole and p-aminosalicylic acid possessed high toxicities for mice.[8] No curative effect was noted when promin in a concentration of 0.5% was included in the diet. This substance was well tolerated by the mice. No improvement was observed when either 0.5% p-aminobenzoic acid or 0.2% methylene blue was added to the diet. The dosage used for these latter agents was that suggested by McLimans and Grant.[9]

---

[7] Barlow, O. W., and Hamburger, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 317.

[8] Bambas, L. L., personal communication.

Discussion. Heilman[10] and Brown and Nunemaker[1] have reported the ineffectiveness of sulfonamides in the prevention of fatal infections when *Streptobacillus moniliformis* was inoculated in mice. In the treatment of arthritis in mice produced by a pleuropneumonia-like microorganism, Findlay *et al.*[11] and Sabin and Warren[12] found sulfonamides, as well as arsenicals and salicylates, to be of no value.

Heilman[10] and Brown and Nunemaker[1,13] reported that a single dose of myochrysine would protect mice from infection and death after inoculation with *Streptobacillus moniliformis*. These investigators, however, injected the myochrysine immediately after the microorganisms were inoculated. Brown and Nunemaker found that if they allowed a chronic arthritis to develop in mice, daily subcutaneous injections of myochrysine for a 2-week period had no effect. The present data are in agreement with this last set of results. On the other hand, Findlay[11] and Sabin and Warren[12] showed certain gold compounds to possess prophylactic and curative value in arthritis in mice caused by pleuropneumonia-like organisms. It should be noted, however, that the arthritis produced by these organisms was a non-suppurative type while in the arthritis that develops in mice following the injection of *Streptobacillus moniliformis,* suppuration is a constant finding.

Brown and Nunemaker[1] reported recovery with myochrysine in 2 cases of rat bite fever in human subjects where there was involvement of the joints and *Streptobacillus moniliformis* was isolated. They stated that although improvement following the use of gold therapy was quite definite, it might have occurred spontaneously. Heilman and Herrell[14] reported 100% protection in mice when penicillin was administered subcutaneously beginning 4½ hours after inoculation of their strain of *Streptobacillus moniliformis*. The infected mice continued to receive penicillin at a level of 1,000 units per day for 7 days. But if penicillin was given for only 5 days, 50% of the mice developed swollen joints. In the present study, although treatment was not begun until 3 days after the first appearance of the arthritis, 60% of the mice recovered if 1,000 units were given 3 times a day for 6 days. Thirty-seven per cent were cured if 10,000 units were administered 3 times a day for 4 days.

The use of penicillin in the treatment of infections due to *Streptobacillus moniliformis* has brought about dramatic recoveries in as short a period as 24 hours, with complete disappearance of the arthritis.[15–17] Altmeier *et al.*,[15] Wheeler,[16] and Weber and Favour[17] reported cases where patients with arthritis due to *Streptobacillus moniliformis* infections promptly recovered with daily intramuscular injections of penicillin.

The most effective therapeutic agent in the treatment of the strain of *Streptobacillus moniliformis* used in the present study was streptomycin. Sprecher and Copeland[18] used this drug in one case where there was joint involvement and in which *Streptobacillus moniliformis* was isolated from the blood. The patient had been treated unsuccessfully with penicillin for 6 days before streptomycin was administered. The third dose of the latter antibiotic brought permanent relief. The authors explained the inefficacy of penicillin in this case by stating that the treatment of the infection caused by *Streptobacillus moniliformis* might depend upon the susceptibility or resistance of the strain involved.

Five other agents were treated in the present study in regard to their effect on joint involvement produced in mice. These substances were investigated because it has been reported that they possess therapeutic activity against some types of microorganisms. The sulfones, promin, and promizole have been reported to be active against some of the acid-fast bacilli.[19-21] P-aminosalicylic acid has also been used to treat experimental tuberculosis.[22] P-aminobenzoic acid has been shown by Snyder *et al.*[23] to be effective in protecting mice infected with a type of epidemic typhus. Methylene blue was shown to be of value in increasing the survival time of mice infected with the etiological agent of Tsutsugamushi disease.[24] None of the above-mentioned agents cured any of the mice of the joint involvement produced by infection with *Streptobacillus moniliformis.*

---

[9] McLimans and Grant, *Science*, 1947, **105**, 181.

[10] Heilman, F. R., *Science*, 1940, **9**, 366.

[11] Findlay, G. M., Mackenzie, R. D., and MacCallum, F. O., *J. Exp. Path.*, 1940, **21**, 13.

[12] Sabin, A. B., and Warren, J., *J. Bact.*, 1940, **40**, 823.

[13] Brown, T. M., and Nunemaker, J. C., *J. Clin. Invest.*, 1940, **19**, 768.

[14] Heilman, F. R., and Herrell, W. R., *Proc. Staff Meet. Mayo Clinic*, 1944, **19**, 259.

[15] Altmeier, W. A., Snyder, H., and Howe, G., *J. A. M. A.*, 1943, **127**, 270.

[16] Wheeler, W. B., *Am. J. Dis. Child.*, 1945, **69**, 215.

[17] Weber, R. A., and Favour, C. B., *Bull. Johns Hopkins Hosp.*, 1945, **77**, 132.

[18] Sprecher, M. H., and Copeland, J. R., *J. A. M. A.*, 1947, **134**, 1014.

Summary. A study of the chemotherapy of the arthritis produced in mice by inoculation with a virulent strain of *Streptobacillus moniliformis* was made. Three days after the animals developed joint involvement, therapy was initiated. Under the conditions studied streptomycin was the most effective agent in curing the joint involvement. Penicillin was also effective but to a lesser degree. No cures were obtained with promin, promizole, sulfathiazole, p-aminobenzoic acid, p-aminosalicylic acid, methylene blue, and two gold compounds (Myochrysine and Solgonal-B).

The authors wish to thank Dr. Carl E. Duffy for his advice and assistance during the course of this study.

---

[19] Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Am. Rev. Tuberc.*, 1942, **45**, 303.

[20] Feldman, W. H., Hinshaw, H. C., and Mann, F. C., *Am. Rev. Tuberc.*, 1944, **50**, 418.

[21] Foget, G. H., Pogge, R. C., and Johansen, F. A., *U. S. Public Health Rep.*, 1946, **61**, 957.

[22] Lehman, J., *Lancet*, 1946, **250**, 15.

[23] Snyder, J. C., Maier, J., and Anderson, C. R., Report to Division of Medical Sciences, National Research Council, December 26, 1942.

[24] McLimans, W. F., and Grant, C. W., *Science*, 1947, **105**, 181.

16470 P

Influence of Diet, Sex, and Testosterone Propionate on the Toxicity of Monocrotaline in Rats.*

Oscar D. Ratnoff and George S. Mirick.

From the Department of Medicine, Johns Hopkins University School of Medicine, Baltimore.

Robertson[1] described the production of hepatic damage in horses fed *senecio,* an observation confirmed in other species.[2-4] Adams and Rogers[5] demonstrated that monocrotaline, an alkaloid derived from *crotalaria,* contained the same active retronecine alkanolamine present in *senecio* alkaloids. Harris, Anderson and Chen[6] observed that mice injected intravenously with monocrotaline died with pulmonary edema, ascites, hydrothorax, and thymic necrosis. There was central hepatic necrosis, hemorrhage and sinusoidal congestion, with little leukocytic reaction. Repeated administration of sublethal doses produced unimpressive hepatic lesions. In

---

[*] This work was supported by a grant from the U. S. Navy Department Office of Naval Research.

[1] Robertson, *Cape Agricultural J.*, May, 1906, quoted by [2].

[2] Theiler, A., U. of S. Africa, Department of Agriculture 5th and 6th Reports of the Director of Veterinary Research, April, 1918, p. 7.

[3] Wilmot, F. C., and Robertson, G. W., *Lancet*, 1920, **2**, 848.

[4] Rosenfeld, I., and Beath, O. A., *Am. J. Clin. Path.*, 1945, **15**, 407.

[5] Adams, R., and Rogers, E. F., *J. Am. Chem. Soc.*, 1939, **61**, 2815.

[6] Harris, P. N., Anderson, R. C., and Chen, K. K., *J. Pharmacol. and Exp. Therap.*, 1942, **75**, 78.

TABLE I.
Survival Time of Rats Injected with Monocrotaline.

Diet	Strain	No.	Sex	Wt, g*	Survival time, days*
A	MacCollum	10	♂	264 (182-327)	18.4 (14-22)
		3	♀	180 (165-196)	22.0 (18-28)
	Total Group A	13			19.2
B	Harvard	7	♂	234 (221-249)	9.0 (6-16)
		4	♀	186 (168-203)	18.7 (8-28)
	MacCollum	11	♂	209 (193-237)	9.3 (6-24)
		7	♀	169 (158-192)	19.3 (8-31)
	Total Group B	29			12.9
C	MacCollum	4	♂	156 (139-179)	18.8 (15-22)
		6	♀	151 (133-167)	20.7 (19-25)
	Total Group C	10			19.9

* Avg; range in parentheses.

rats, monocrotaline prolonged the prothrombin time.[7]

In the course of studies on dietetic hepatic pathology in rats, the toxicity of monocrotaline was investigated.

Methods. Hooded rats of 2 strains were individually housed after weaning, allowed water as desired, and fed one of the following diets: Diet A: Purina Dog Chow as desired for 5 to 13 weeks. Diet B:[8] Casein, 4%; dried brewer's yeast, 16%; corn starch, 71%; cod liver oil, 1%; peanut oil, 5%; salt mixture,[9] 3%; plus thiamine chloride, 20 μg, riboflavin, 25 μg, pyridoxine, 20 μg, and calcium pantothenate, 120 μg per rat daily. Each rat received 11 g daily for 8 to 24 weeks, and then 8 g daily for 2 to 6 weeks. Diet C: Diet B, 8 g daily for 7 to 11 weeks. Each rat was then injected intraperitoneally every second day with 46 mg of 1% buffered monocrotaline† per kilo (half the intravenous LD_{50} dose). During this period, rats on Diet A were fed as desired and those on Diets B and C received 8 g daily.

Results. (1) *Pathologic Effects of Monocrotaline.* In brief, the injection of monocrotaline resulted in apathy, anorexia, weight loss, dark urine, epistaxes, purpuric paws and occasional terminal paraplegia. The gross pathologic findings included bloody peritoneal and pleural fluid, splenic enlargement, hemorrhagic livers, thymus and mesentery and, with longer survival, yellow stained tissues, firm red lungs, and right auricular dilatation. The histopathology included disruption of lymphoid follicles in the spleen, thymus and lymph nodes; in the liver, early, severe central hemorrhage was followed by central and midzonal necrosis without leucocytic reactions.

(2) *Effect of Diet and Sex on Survival Time of Rats Injected with Monocrotaline.* The effect of repeated injections of monocrotaline in rats is demonstrated in Table I and Fig. 1.

The data suggest that male rats of 2 strains fed a moderately low protein diet (Diet B) were more susceptible to monocrotaline than pair-fed females, or males and females fed a balanced diet (Diet A). The males were more susceptible only if they were fed sufficient diet so that they weighed more than pair-fed females. When the low protein diet was limited so that the sexes were of equal weight (Diet C) both sexes were equally resistant to monocrotaline. The rats, 10 to 14 weeks old at the time of the first injection, never showed gross evidence of sexual maturity. Half of them received injections of mixed methionine 60 mg and menadione, 5 mg daily without effect on survival time.

(3) *Effect of Testosterone Propionate on Survival Time of Rats Injected with Mono-*

---

[7] Rose, C. L., Fink, R. D., Harris, P. N., and Chen, K. K., *J. Pharmacol. and Exp. Therap.*, 1945, **83**, 265.

[8] MacCollum, F. O., and Miles, J. A. R., *Lancet*, 1946, **1**, 3.

[9] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

† Monocrotaline was obtained through the courtesy of Dr. Roger Adams and Dr. K. K. Chen.

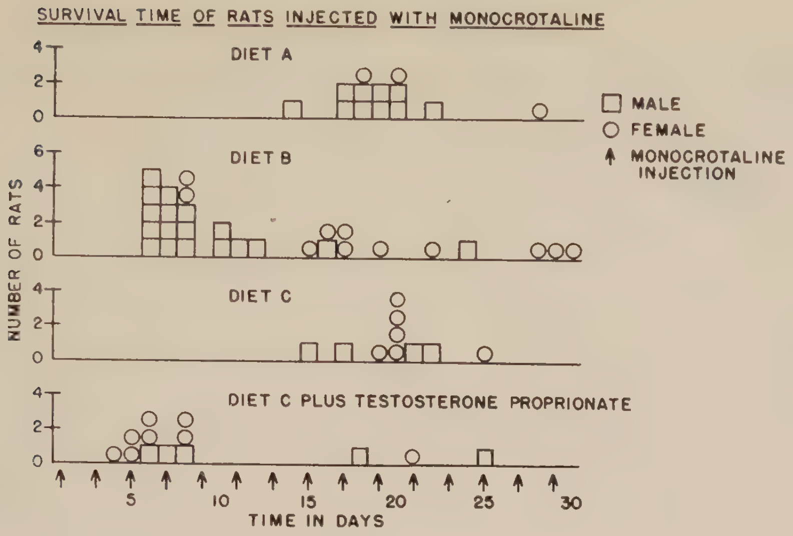


Fig. 1.
The survival time of rats injected intraperitoneally every second day with 46 mg per kilo of monocrotaline. The diets are described in the text.

TABLE II.
Influence of Testosterone Propionate upon Survival Time of Rats Injected with Monocrotaline.

Regime	No. of rats	Sex	Wt † g*	Survival time, days*
Control	4	♂	139 (128-146)	19.2 (14-25)
	9	♀	137 (115-161)	17.3 (13-20)
Testosterone propionate	5	♂	147 (128-167)	12.8 (6-25)
	8	♀	149 (133-160)	7.9 (4-21)

* Avg; range in parentheses.
† At time of first monocrotaline injection.

crotaline. Thirteen rats which had been fed Diet C for 6 to 8 weeks were injected subcutaneously with 2.5 mg of testosterone propionate in sesame oil (Rare Chemicals, Inc.) daily for 2 weeks, and then with monocrotaline as described, while the testosterone propionate was continued. At the same time 13 control rats of the same litters and on the same diets were injected with monocrotaline alone. Injection of testosterone propionate increased the susceptibility to monocrotaline of male and female rats fed low protein diets so that there was no difference in weight between the sexes. (Table II).

Discussion. The extensive hepatic destruction observed leaves little doubt of the importance of hepatic failure in the death of rats injected with monocrotaline. György and Shipley[10] observed that female rats fed low protein diets were more resistant than males to carbon tetrachloride, and that estrogenic substances, particularly in combination with methionine, were lipotropic.[11] The present study suggests that the presence of male hormones may accentuate the toxicity of monocrotaline. It is noteworthy that Blackman, Thomas, and Howard[12] have observed histologic changes in the livers of testosterone-treated dogs.

Summary. Male rats fed a moderately low protein diet were more susceptible than females, or than both sexes on a normal diet, to monocrotaline, an alkaloid causing hemorrhagic hepatic damage and other lesions. Rats of both sexes on still more deficient diets were as resistant as normal rats. Testosterone propionate increased the toxicity of monocrotaline for both sexes on the more deficient diet. These experiments suggest that the greater susceptibility of male rats to monocrotaline may be due to the metabolic effects of male sex hormones.

---

[10] György, P., Seifter, J., Tomarelli, R. M., and Goldblatt, H., *J. Exp. Med.*, 1946, **83**, 449.

[11] György, P., Rose, C. S., and Shipley, R. A., *Arch. Biochem.*, 1947, **12**, 125.

[12] Blackman, S. S., Jr., Thomas, C. B., and Howard, J. E., *Bull. Johns Hopkins Hosp.*, 1944, **74**, 321.

We wish to acknowledge the valuable technical assistance of Mrs. Betty Jean Stetson.

16471

Total 17-Ketosteroid Excretion in Normal and Ovariectomized Rabbits.*

J. DeKoning, B. Krichesky, S. J. Glass.†

From the Department of Zoology, University of California, Los Angeles.

The excretion levels of the 17-ketosteroids in urine have been studied extensively in humans. Comparisons of the levels of normal and castrate females vary; some workers have reported no significant difference after ovariectomy,[1,2] while others reported below normal levels, but with some overlapping.[3] Friedgood[4] observed a drop following castration, then a rise to normal. In a study on 2 Rhesus monkeys, Dorfman[5] reported a marked rise immediately after gonadectomy, returning to normal by the 22nd day in 1 animal while the other showed only a slight decrease during the first 5 days.

Because the environmental conditions of experimental animals are more easily controlled than those of human subjects, it was considered that the animal affords better opportunity for study of excretion levels under experimental conditions. This report deals with the normal and castrate levels of the total 17-ketosteroids in female rabbit urine.

Materials and Method. All animals were of the New Zealand strain, varying in age between 5 months and 2½ years. They were fed complete rabbit pellets with the exception of one change which is discussed later. The animals were housed in separate cages over screened trays which connected collecting flasks. When bilaterally ovariectomized, the animals were allowed at least a 17- to 21-day recovery period before collections were begun. Two animals had been ovariectomized one year previously.

The urine samples were collected for 48-hour periods over 5 ml hydrochloric acid, and were stored at 5°C until used. They were extracted according to the method of Pincus,[6] using a volume of 250 or 300 ml of urine. The crude neutral fractions were treated with Girard "T" in the cold.[6] Colorimetric assay was carried out in absolute alcohol[7] using a Beckman spectrophotometer, with a dehydroisoandrosterone standard.‡ The reaction color obtained with rabbit urine extracts is similar

---

* Aided by a research grant from Ayerst, McKenna and Harrison, Ltd., and the Board of Research, University of California.

† The authors wish to express their thanks to Mr. Henry Rubin for his valuable technical assistance.

[1] Fraser, R. W., Forbes, A. P., Albright, F., Sulkowitch, H., and Reifenstein, E. C., *J. Clin. Endocrin.*, 1941, **1**, 234.

[2] Hamblen, E. C., Ross, R. A., Cuyler, W. K., Baptist, M., and Ashley, E., *Endocrin.*, 1939, **25**, 491.

[3] Callow, N. H., Callow, R. K., and Emmens, C. W., *J. Endocrin.*, 1940, **2**, 88.

[4] Friedgood, M., *A.A.A.S. Symposium: The Chemistry and Physiology of Hormones*, 1944, pp. 195-202.

[5] Dorfman, R. I., Horwitt, B. N., Shipley, R. A., Fish, W. R., and Abbott, W. E., *Endocrin.*, 1947, **41**, 470.

[6] Pincus, G., *J. Clin. Endocrin.*, 1945, **5**, 291.

[7] Talbot, N. B., Butler, A. M., and MacLachlan, E., *J. Biol. Chem.*, 1940, **132**, 595.

‡ Crystalline dehydroisoandrosterone was generously supplied by Ciba Pharmaceutical Products, Inc., and by Schering Corporation.

TABLE I.
17-Ketosteroid Excretion and Urine Volumes in Normal Female Rabbits.

Group	Animal	No. of runs	Avg 17-ks. mg/48 hr	Avg urine vol. ml/48 hr
1	A	13	1.50	544
	B	8	1.36	275
	C	11	1.50	358
	D	6	1.38	344
	Total	38	1.45	400
			S.D. = 0.07	
2	I	9	2.56	441
	J	6	4.22	592
	K	7	3.53	488
	L	5	5.79	555
	M	12	4.45	593
	N	12	3.72	572
	O	6	3.33	336
	P	6	4.00	444
	Total	63	3.88	515
			S.D. = 0.89	

TABLE II.
17-Ketosteroid Excretion and Urine Volumes in Castrate Female Rabbits.

Group	Animal	No. of runs	Avg 17-ks. mg/48 hr	Avg urine vol. ml/48 hr
1	E	7	1.34	266
	F	6	1.29	312
	G	5	1.89	318
	H	6	1.84	456
	Total	24	1.56	335
			S.D = 0.27	
2	I	6	4.44	275
	J	9	4.95	449
	K	7	4.61	374
	A	6	2.77	314
	Total	28	4.29	365
			S.D. = 0.85	

to that developed with human urine extracts. All blanks and extracts were assayed in triplicate. A total of 101 normal and 52 castrate urine samples were run in this manner.

Reagents. Ether for extractions, U.S.P. grade, was treated with ferrous sulfate and thoroughly washed with distilled water. Other reagents were of C.P. grade. The absolute ethanol was further dried and purified. *m*-dinitrobenzene was recrystallized until nearly colorless.

Results and discussion. The results are shown in Tables I and II. The amount of the 17-ketosteroids excreted by 4 normal intact rabbits is, for Group 1, 1.45 ± 0.07 mg, for 4 castrate animals the corresponding level is 1.56 ± 0.27. The normal for the 8 animals of Group 2 is 3.88 ± 0.89 mg, with the castrate level being 4.29 ± 0.85 for 4 animals. Both normal and castrate levels are shown for animals J, K, and I.

Although the average difference in 17-ketosteroid excretion appears slightly higher in castrate than in normal rabbits, a statistical analysis shows that there is no significant difference between them.§ These data are in

---

§ $$\frac{M_{\text{normal}} - M_{\text{cast.}}}{\text{S.E.}_{M_n - M_c}} \,/\, 3 \quad \begin{matrix}(\text{Group } 1 = 2.1)\\(\text{Group } 2 = 2.0)\end{matrix}$$

agreement with those presented by Fraser *et al.*,[1] Hamblen *et al.*,[2] and Callow *et al.*,[3] and contrary to the findings of Friedgood.[4]

It will be noted that in both normal and castrate animals (Tables I and II) there are two groups of animals, each showing different values. The only reason for division into 2 groups was: (a) in both Tables I and II the animals of Group 1 were older than those in Group 2 (1 to 2½ years as compared with 5 to 6 months); (b) the complete rabbit pellets fed were changed abruptly and unintentionally so that all animals in Group 1 received one brand of pellets, while those of Group 2 received a different brand (this change was instituted by the animal caretakers). It was for this reason that the animals were considered separately, and not because of their ketosteroid values.

The average urine volumes are also shown in Tables I and II. It was found that there is no statistical correlation between urine volume and 17-ketosteroids excretion in normal or castrate animals. (Correlation factor r = —0.119 ± 0.095 for normals, —0.118 ± 0.136 for castrates, showing an insignificant negative correlation). This is in agreement with work done by Kimmeldorf on male rabbits,[8] as well as by Bachman *et al.*,[9] and Pincus[10] on humans.

Summary. 1. Total 17-ketosteroid urinary levels have been determined for normal and ovariectomized rabbits. There is no statistical difference between them. 2. The ratio between urine volume and 17-ketosteroid excretion has been examined statistically and no correlation found.

---

[8] Kimmeldorf, D., (unpublished data obtained in these laboratories).

[9] Bachman, C., Leekley, D., and Winter, B., *J. Clin. Endocrin.*, 1941, **1**, 142.

[10] Pincus, G., *J. Clin. Endocrin.*, 1943, **3**, 195.

16472

Effect of Orally Administered Sodium Estrone Sulfate on Total 17-Ketosteroid Excretion in Female Rabbits.*

J. DeKoning, B. Krichesky, and S. J. Glass.†
From the Department of Zoology, University of California, Los Angeles.

The effects on the total 17-ketosteroid excretion after estrogen administration have not been studied extensively. Hamblen and his co-workers[1] studied the change in 17-ketosteroid excretion titers after estrogen administration to women in the climacterium, those complaining of hypoovarianism or functional uterine bleeding.

This study was undertaken to determine the effect, if any, of the oral administration of sodium estrone sulfate (Premarin‡) on the total 17-ketosteroid excretion in normal and ovariectomized rabbits.

Materials and methods. Six female rabbits of the New Zealand strain, 4 normal and 2 castrate, were used for this experiment. The age of the animals varied from 7 months to 2-2½ years. Animals V and VI were bilaterally gonadectomized 2 months before being used in this experiment. All animals were kept in separate cages and fed complete rabbit pellets.

The urine was collected for 48-hour periods over 5 ml HCl. It was extracted and purified with Girard "T" according to Pincus,[2] and

---

* Aided by a research grant from Ayerst, McKenna, and Harrison, Ltd., and the Board of Research, University of California.

† The authors wish to express their thanks to Mr. Henry Rubin for his valuable technical assistance.

[1] Hamblen, E. C., Pattee, C. J., and Cuyler, W. K., *Endocrin.*, 1940, **27**, 734.

‡ Generously supplied by Ayerst, McKenna and Harrison, Ltd.

[2] Pincus, G., *J. Clin. Endocrin.*, 1945, **5**, 291.

TABLE I.
Effect of Orally Administered Sodium Estrone Sulfate on Total 17-Ketosteroid Excretion in Female Rabbits.

Animal	Normal 17-ks. mg/48 hrs	Dose of Estrogen (mg)	Mg 17-ks. after Na estrone sulfate admin. 1st 48 hr	2nd	3rd	4th	Avg
I (Normal)	2.73 (6)*	1.25	1.62	3.47	3.30	—	2.80
		0.63	1.68	1.70	1.35	—	1.58
II ”	2.43 (6)	0.63	2.59	4.61	2.99	—	3.39
		0.63	1.76	1.82	1.04	—	1.54
III ”	1.91 (6)	0.63	1.06	3.60	3.67	—	2.78
		0.63	2.90	1.77	2.75	—	2.47
IV ”	3.92 (9)	2.50	4.24	3.18	6.10	4.18	4.42
V (Castrate)	4.94 (6)	2.50	2.30	4.09	4.47	5.34	4.05
VI ”	4.44 (5)	2.50	3.51	2.58	3.63	4.04	3.44

* The figure in parenthesis represents the number of 48-hour collections.

assayed colorimetrically[3] on a Beckman spectrophotometer. A total of 38 collections were treated in this manner. The reagents were purified as described in a previous paper.[4]

The estrogen was administered, in liquid form by stomach tube and in dry form as a pill, inserted to the back of the mouth. It was administered in one dose (0.63 mg to 2.50 mg) and immediately following, the urine was collected for 3 or 4 consecutive 48-hour periods. Animals I, II, and III received a second dose one month later, and 3 determinations were made as before.

Results and discussion. All pertinent data, including 17-ketosteroid values, dosage, etc., are included in Table I. An examination of the results shows (1) statistically there is no significant change from the normal in the 17-ketosteroid excretion levels after administration of sodium estrone sulfate for at least 192 hours with a dose varying from 0.6 mg to 2.5 mg; (2) the data on excretion levels for the two castrate animals employed show no differences before and after estrogen feeding within the limits of the experiment; (3) although there is considerable variation between consecutive excretion titers on individual animals after estrone administration, there is no consistent trend for the 17-ketosteroids to increase or decrease.

Although these observations are not in accord with the reports of Hamblen's group;[1] it must be pointed out that his patients, for various reasons, all showed abnormally high 17-ketosteroid titers; and even with the decrease following estrogen therapy, in none of the cases did titers fall below what he considered the average normal excretion for healthy women.

Summary. The oral administration of sodium estrone sulfate in doses ranging from 0.6 mg to 2.5 mg to normal female rabbits or 2.5 mg to castrate female rabbits has been found to have no effect on the total urinary 17-ketosteroid excretion over a period of 192 hours.

---

[3] Talbot, N. B., Butler, A. M., and MacLachlan, E., *J. Biol. Chem.*, 1940, **132**, 595.

[4] DeKoning, J., *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 320.

16473

Species Differences in the Reaction of the Mammalian Skeleton to Estrogens.*

MARSHALL R. URIST, ANN MARIE BUDY, AND FRANKLIN C. MCLEAN.
From the Department of Physiology, University of Chicago, Chicago, Ill.

In recent years it has been observed that the bones increase in density in immature mice and rats treated with large doses of estrogen. It has not been adequately emphasized, however, that the bone changes in mice described by Gardner and Pfeiffer,[1] are very different from the skeletal reaction in rats described by Follis and Day.[2] Nor is it generally appreciated that many other mammals, including man, react differently from either the rat or mouse.[3-5] This communication is the introduction to an extensive survey of the reactions of the skeleton in mice, rats, rabbits, hamsters, guinea pigs, cats, and dogs treated with 7 different natural estrogens and 4 different synthetic estrogens. For the sake of brevity, only the conclusions related to the above title will be stated from gross, x-ray, and histological examinations of the bones. Undecalcified sections, prepared according to technics previously described,[6,7] were used in addition to standard decalcified histological sections.

Mice. Large doses of α-estradiol benzoate or diethylstilbestrol, approximately 0.5 mg per week, produced endosteal bone formation in mice somewhat like that observed in estrogen-treated birds.[6] This reaction was produced

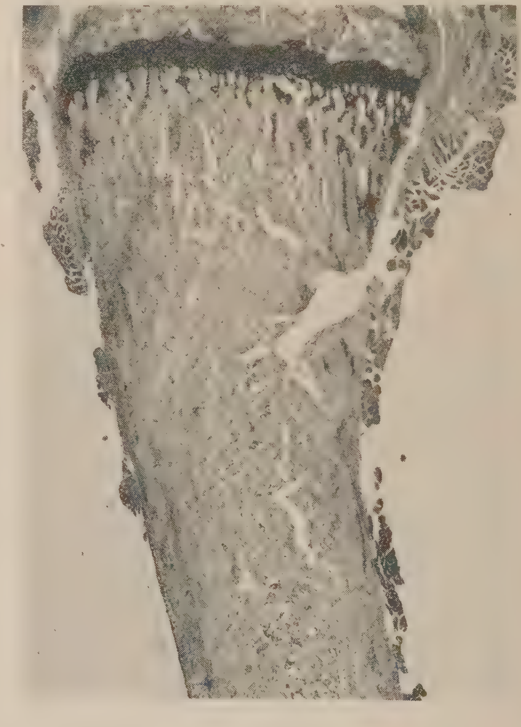


FIG. 1.
Photomicrograph of the upper end of the tibia of an immature mouse, approximately 7 weeks of age, treated with 1.0 mg of alpha-estradiol benzoate in 2 weeks and sacrificed 1 week later. The metaphysis is increased in density and the marrow cavity is filled with a network of new bone. This is the result of two processes, stimulation of endosteal or intramedullary new bone formation, and inhibition of bone resorption. Hematoxylin-eosin and azure-II stain.

---

* These investigations were aided by grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service; from the Josiah Macy, Jr., Foundation; and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Crystalline steroid hormones were provided by Dr. Kenneth W. Thompson of Roche-Organon, Inc., Dr. E. C. Reifenstein of Ayerst, McKenna, and Harrison, Ltd., and Dr. Erwin Schwenk of the Chemical Research Division of the Schering Corporation.

[1] Gardner, W. U., and Pfeiffer, C. A., *Physiol. Rev.*, 1943, **23**, 139.

[2] Day, H. G., and Follis, R. H., *Endocrinol.*, 1941, **28**, 83.

[3] Sutro, C. J., and Pomerantz, B. S., *Arch. Path.*, 1942, **33**, 305.

[4] Koneff, A. A., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1946, **94**, 169.

[5] Urist, M. R., Bell, R., Jr., and Voshell, A. F., unpublished studies on children with Osteogenesis Imperfecta.

[6] Bloom, M. A., McLean, F. C., and Bloom, W., *Anat. Rec.*, 1942, **83**, 99.

[7] Bloom, W., Bloom, M. A., and McLean, F. C., *Anat. Rec.*, 1941, **81**, 443.

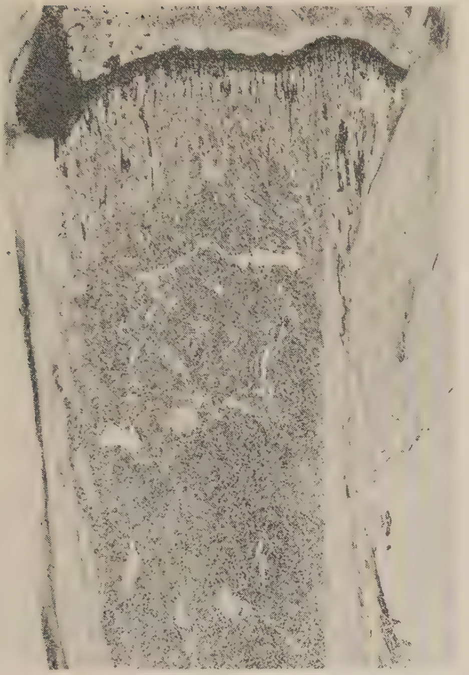


FIG. 2.
Photomicrograph of the upper end of the tibia of a rat, 12 weeks of age. Note the wide intervals between bone trabeculae in the area below the hypertrophic cells of the epiphyseal plate. Note the length of the new bone trabeculae of the primary spongiosa. The new bone formed in endochondral ossification contains a core of calcified cartilage matrix. Hematoxylin-eosin and azure-II stain.

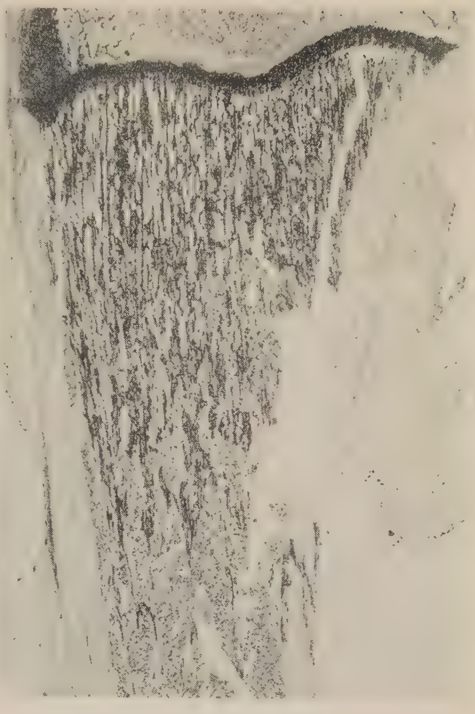


FIG. 3.
Photomicrograph of the upper end of the tibia of a litter-mate of the rat shown in Fig. 2, after 0.56 mg of alpha-estradiol benzoate in a period of 8 weeks. Note the metaphysis is approximately 5 times longer than normal. Because of the failure of resorption of most of the bone formed by endochondral ossification, the trabeculae extend down into the medulla and each one contains a core of deeply-staining calcified cartilage matrix. There is no endosteal new bone, as found in the reaction of the skeleton of the mouse, shown in Fig. 1. Hematoxylin-eosin and azure II.

consistently in animals between 1 and 6 months of age. The process began in the metaphyses of the rapidly growing long bones at 10 days and extended along the shaft until, at 10 to 12 weeks, the entire medulla was filled with new bone (Fig. 1). The intramedullary or endosteal new bone formation was not as widespread in treated mice as in birds[7] during the preovulatory phase of the egg-laying cycle. In birds, osteogenesis was rapidly initiated in the endosteum all along the inner shaft. In mice, osteogenesis progressed only by extension from the metaphyses, but the new bone formed similarly at the lines of apposition of osteoblasts and transforming bone marrow cells. The histological picture suggested that the reticular cells of the marrow stroma are drawn into the osteogenic process and become osteoblasts. Bone resorption was markedly inhibited, osteoclasts were rarely formed, and all the medullary new bone appeared to remain as long as the injections of estrogen were continued.

Rats. Large doses of α-estradiol benzoate or diethylstilbestrol (0.14 to 0.5 mg per week) repressed the resorption of the new bone produced in endochondral ossification. There was no appreciable effect upon bone formed in intramembranous ossification, except perhaps a non-specific stunting of the growth of the whole animal.

The effect of estrogen on the skeleton of the rat is specifically upon the process of resorption of cartilage matrix and new bone formed at the epiphyseal lines. Two effects are seen in normal bone resorption: the "thinning out" of the spongiosa and the control of the normal length of the trabeculae. Estrogen prevented both the normal thinning and shortening of the metaphysis which is seen in young growing animals with rapid turnover of the body calcium stores. The metaphyses increased in length in young rats because there was no bone resorption at the ends of the trabeculae. Osteoclasts were almost completely absent from the scene. After 8 weeks of treatment, the bone trabeculae were everywhere filled with a core of unresorbed cartilage matrix, both in the secondary spongiosa near the shaft and in the new cancellous bone at the epiphyseal lines.

The overall increase in the length of the spongiosa was proportional to the period of treatment. Growth of the bones in length was retarded by estrogen, particularly when large doses were given to weanlings for 5 weeks. With moderate doses there was less inhibition of growth and the length of the metaphyses was greater than in animals treated with large doses for the same period of time.

The increase in density of the spongiosa, or the number of bone trabeculae in the metaphysis, was directly proportional to the dose of estrogen. On large doses, the cancellous bone became so dense that it was indistinguishable from the compact bone of the shaft in the roentgenograms. In rats these effects were produced only between 1 and 6 months of age and were most pronounced during the period of rapid growth between 1 and 3 months of age.

Other Species. Previous experimental investigations dealing with the effects of estrogen upon the skeleton of other mammals have been limited to the treatment of hamsters,[4] guinea pigs,[8] and puppies[3] with relatively small doses of estradiol benzoate or diethylstilbestrol. We have treated these and other species with doses of estrogen as large as the animal would tolerate. The bone changes were quite different from those described above in mice and rats. In hamsters, guinea pigs, rabbits, kittens, and puppies, proliferation of epiphyseal cartilage and new bone formation were suppressed. The overall result of treatment was that the growth of the long bones was stunted and the metaphyses were reduced to one-half or one-fourth the normal length. There was also an appreciable reduction in the number of osteoblasts and bone trabeculae in some species, *i.e.*, immature rabbits treated with 30 to 60 mg of diethylstilbestrol within 4 weeks. In many species the marrow showed widespread degenerative changes and formation of colloid cysts. The histological picture in general suggested nonspecific repression of all cellular activity with none of the stimulating action of estrogen upon osteogenesis as seen in the mouse or none of the inhibiting effect of estrogen on cartilage resorption in the rat.

Summary. Estrogenic steroid hormones stimulate endosteal bone formation and inhibit bone resorption in mice. In young growing rats, the same substances do not produce endosteal bone but interfere with endochondral ossification by inhibiting resorption of cartilage matrix and new bone. In other rodents, such as hamsters, guinea pigs, and rabbits, and also in cats and dogs, there is no apparent specific effect of estrogens upon formation of bone.

---

[8] Sutro, C. J., and Pomerantz, L., *Arch. Surg.*, 1939, **39**, 992.

16474

Fate of Food Introduced Directly into the Fetal Stomach.*

J. Francis Hartmann and L. J. Wells.

From the Department of Anatomy, University of Minnesota, Minneapolis, Minn.

The literature on the human fetus contains records of several studies devoted to the time of origin of function in the digestive system. Thus, the presence of gastric glands at the 4th and 5th month has been reported[1,2] and various enzymes have been identified at about the 4th month.[3-6] From such observations as these, it has been inferred that the fetus is prepared to carry on various digestive processes in advance of birth. Also, as is well known, premature infants may survive. It is, however, an entirely different question as to whether the fetus can actually digest food during the time when its nutrition is being supplied via the placenta.

Recently, methods of experimenting directly upon fetal rats have been devised. These methods involve subjecting fetuses to surgical procedures and subsequently returning them to the maternal abdominal cavity for varying periods of time, the placental circulation being maintained intact throughout.[7]

As a means of securing direct evidence concerning antenatal digestive abilities, these operative procedures were utilized in the present series of exploratory experiments.[†] A total of 73 rat fetuses, with placental circulation kept intact, were subjected to laparotomy and subsequent return to the maternal abdominal cavity.[8] Of these, 48 lived to autopsy, giving an overall survival value of approximately 66%. The fetuses were divided into three dietary series.

In the first group (Table I), raw beef or venison, in amounts ranging from 1.0 to 3.5 mg, was inserted directly into the exteriorized fetal stomach through a minute perforation in the stomach wall. The latter was then closed with a single stitch, the fetal abdominal wall was sutured, and the fetus returned to the maternal abdominal cavity. At autopsy, the stomach contents were examined, and muscle fibers were found to be microscopically recognizable as late as 53 hours after operation. It will be noted in Table I that in the 2 fetuses examined at 47 hours, the presence of muscle fibers at autopsy was doubtful. This is due to the fact that in these 2 instances, a microscopic analysis of the stomach contents was not carried out. In all cases, however, there was a discrete mass present in the stomach, representing the remains of the meat originally introduced. The mass was somewhat softer in consistency than when introduced, and upon teasing it under the microscope, the changed consistency was found to be due to a softening of the connective tissue elements. The muscle fibers themselves gave no morphological evidence of having been attacked by digestive enzymes. Ligation of the fetal esophagus did not seem to influence the results.

In the second series (Table II), cow's milk or human milk was injected directly into the exteriorized stomach. The first group represents fetuses returned to the maternal abdominal cavity after injection, the second group

---

* Aided by grants from Ciba Pharmaceutical Products, Inc., and from the Graduate Research Fund of the University of Minnesota.

[1] Kolliker, A., *Entwicklungsgeschichte des Menschen und der höheren Thiere,* Wilhelm Engelmann, Leipzig, 1879.

[2] Scott, G. H., *Am. J. Dis. Child.,* 1925, **30**, 147.

[3] Ibrahim, J., *Biochem. Z.,* 1909, **22**, 24.

[4] Keene, M. F. L., and Hewer, E. E., *Lancet,* 1929, **1**, 767.

[5] Tachibana, T., *Jap. J. Obst. and Gynec.,* 1927, **10**, 27.

[6] Tachibana, T., *Jap. J. Obst. and Gynec.,* 1928, **11**, 92.

[7] Wells, L. J., *Anat. Rec.,* 1946, **94**, 530.

[†] The observations reported here have been abstracted,[8] and it seems desirable to present the data in fuller detail since it is unlikely that additional new information could be obtained without first modifying the experimental approach.

[8] Hartmann, J. F., and Wells, L. J., *Anat. Rec.,* 1948, **100**, 673.

TABLE I.
Introduction of Meat into Fetal Stomach.

Hrs in stomach	No. of fetuses	Exp. begun, prepartum age in days	Amt, mg	Muscle fibers present at autopsy
6	1	22	3.5	+
6	1	22	3.2	+
23	1	22	3.0	+
23	1	22	1.5	+
47	2*	20	1.4	±
49	1*	20	0.8	+
52	1	20	1.8	+
53	1	20	1.4	+

* Esophagus ligated.

TABLE II.
Injection of Milk into Stomach.

Hrs in stomach	No. of fetuses	Exp. begun prepartum age in days	Amt. injected, cc Cow's milk	 Human milk	Stomach contents at autopsy
			A. Fetuses.		
¼	1	22	.05		Flocculent curd
¼	1	22	.03		” ”
3	1	22	.05		Soft discrete curd
7	1	22	.05		” ” ”
7	1	22	.05		Hard curd
23	1*	21	.05		Flocculent curd
27	1	21	.045		Whitish mucoid mass
29	1*	21	.04		Trace of flocculent curd
49	1	20	.025		Flocculent curd
52	1	20		.05	Whitish mucoid mass
52	1	20		.05	Flocculent curd
52	1	20		.03	” ”
53	1	20		.02	Whitish mucoid mass
			B. Caesarian Newborns.		
½	2	22	.05		Fluid
¾	1	22	.03		Flocculent curd
¾	1	22	.05		Fluid
¾	1	22	.05		Soft curd
2	1	22	.03		Firm ”
2	1	22	.05		Hard ”
7	1	22	.05		Fluid
15	2	22	.05		Hard curd
19	1	22	.10		Soft ”
23	1	22	.10		Curd firm at cardia
30	1	22	.075		Soft curd

* Esophagus ligated.

comprises Caesarian newborns taken on the last day of gestation and kept at room temperature for the lengths of time indicated in the first column. At autopsy, the stomachs of fetuses examined shortly after operation contained flocculent curds. Curds found 7 hours postoperatively were soft to hard, those examined at 23 hours being again flocculent in character. Traces of flocculent curd, however, were still present in the cardiac region of the stomach at 49 and 52 hours after injection. From observation of the stomach contents in these animals, one gains the impression that curds begin to form soon after the milk is injected, that they later solidify, and still later soften again. The softening is more noticeable at the pyloric end of the stomach. Whether or not this indicates an actual progression of digestive activity in the fetal stomach is not entirely clear from the present data, and chemical analyses of the stomach contents will undoubtedly supply additional evidence on this point. In some instances, no discrete curd was found at

TABLE III.
Normal Newborns Allowed to Nurse.

Hr in stomach	No. of animals	Stomach contents at autopsy
13	1*	Empty; probably had not nursed
15	1*	Soft curd
16	1	” ”
21	1	” ”
27	1	Trace of curd
37	1	Empty

* Kept at 37°C.

TABLE IV.
Injection of 10% Uncooked Corn Starch into Fetal Stomach.

Hrs in stomach	No. of fetuses	Exp. begun prepartum age in days	Amt in cc	Iodine test at autopsy			
				Rectum	Ileum	Duodenum	Stomach
21	1*	22	.05			+	+
22	1*	22	.05			+	+
23	1*	22	.05	—		+	+
24	1*	22	.05	—		+	+
24	1*	22	.01		+	+	+
27	1	21	.02	—		+	+
27	1	21	.02	—		—	—
27	1	21	.01	—		—	—
30	1	21	.01	—		—	—
30	1	21	.05	—		—	—
49	2	20	.015	—		—	—

* Caesarian newborns.

autopsy, but instead the stomach contained an abundance of mucoid material in which the milk was dispersed, so that no definite curd was observable. The mucoid mass probably represents maternal peritoneal fluid swallowed by the fetus and partially coagulated in the stomach, after being mixed with mucus of fetal origin.

The emptying time of the stomach in operated animals was unexpectedly long,[9] and the delay in emptying is no doubt due in part to operative trauma. In an attempt to evaluate the importance of trauma in delaying the emptying time, a number of normal newborns were allowed to nurse, then separated from the mother, and autopsied from 13 to 37 hours later (Table III). Traces of ingested milk were found in the stomach at 27 hours in one animal, but not in the one examined at 37 hours. The curds were in all cases softer than the hardest curds formed from either cow's milk or human milk. It appears probable that under normal postnatal conditions, the supply of milk in the stomach is constantly replenished by nursing, so that complete emptying never actually takes place. However, the fact that the stomach of the normal newborn rat does not empty completely until after 27 hours, plus the fact that the fetuses injected on the 20th prepartum day were in an earlier stage of development than the newborns, leads to the belief that operative trauma is not the sole cause of the delay in emptying time observed in the fetuses.

The third dietary series (Table IV) received varying amounts of a 10% suspension of uncooked corn starch, administered in the same manner as the milk. At autopsy, the stomach and parts of the intestine were removed to separate glass slides, treated with iodine, and examined under the microscope for the presence of starch grains. Up to 27 hours after injection, blue-stained starch grains were found in the stomach and small intestine, but after 27 hours, none were detectable by the iodine test. The possibility suggests itself that digestion had proceeded at least to the stage of achroodextrins. Since, however, the conditions of the experiment did not preclude bacterial digestion, this remains only a possibility, pending more elaborate tests of the gastrointestinal contents to show whether or not the disappearance of the starch grains is attributable to the action of amylolytic enzymes of the fetus itself.

---

[9] Windle, W. F., and Bishop, C. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 2.

In conclusion, the data show that in the fetal rat with the placenta still functioning, the stomach empties more slowly than might have been anticipated, and they suggest that the fetus has the ability to digest in part such introduced foods as milk and starch. It would seem that the ability to digest meat is less well developed or absent. This exploratory study demonstrates the feasibility of obtaining additional direct experimental data on the digestive abilities of the fetus.

16475

Carcinogenic Effect of Sulfonamides.*

ALF T. HAEREM. (Introduced by G. F. Dick.)
From the Department of Medicine, University of Chicago.

Early workers in the field appreciated the possibility of sulfonamide carcinogenesis because of the similarity in structural formulae between the sulfonamides and known carcinogenic agents of the aniline dye group. Marshall suggested investigation of the possible carcinogenic effects of sulfonamides and Lewis[1] subsequently reported that the injection of sulfanilamide in olive oil was not followed by the appearance of tumors in the strain of mice used. Similar results were later reported when other workers repeated these experiments.[2] Using a slightly different strain of mice and material it was later reported[3] that spindle cell sarcomata developed at the site of injection of 15 mg of sulfanilamide in lard in a small percentage of Swiss albino mice so injected. Since that report the entire experiment has been repeated. Using 20 mice of the same strain, under the same conditions, the same amount of sulfanilamide was injected. Eight (40%) of the group developed a spindle cell sarcoma at the injection site. The mice developing tumors survived from 54 to 269 days (average was 145 days after injection). A similar number of mice injected with lard alone developed no tumors.

In an attempt to further evaluate the sulfonamides in regard to carcinogenic properties, these studies were repeated on albino rats. One group was injected with sulfanilamide in lard and this was compared with a group injected with a known carcinogenic agent (1:2:5:6 dibenzanthracene) in lard. Controls consisted of one group in which lard alone was injected and one group in which no injections were made. Similar observations were made on rats injected with sulfapyridine.

Group I. Sulfanilamide injections (test animals). Two injections of 0.015 g sulfanilamide were given to each of 10 rats. The first injection was made at the age of 14 weeks into the left groin and the second at 23 weeks into the right groin. Nine months after the first injection one of the rats had developed a huge mass in the left groin. Biopsy revealed the tumor to be a spindle cell sarcoma. The tumor was readily transplanted to another rat. Autopsy one month later on the original rat revealed a frankly invasive tumor which had metastasized to both lungs. The cell structure of the original tumor and metastases was identical. Both were composed almost entirely of spindle cells. Mitotic figures were

---

* Aided by a grant from the Fenger Fund.

[1] Lewis, M. R., *Am. J. Cancer,* 1938, **34**, 431.

[2] Zamecnik, P. C., and Koletsky, S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 391.

[3] Haerem, A. T., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 536.

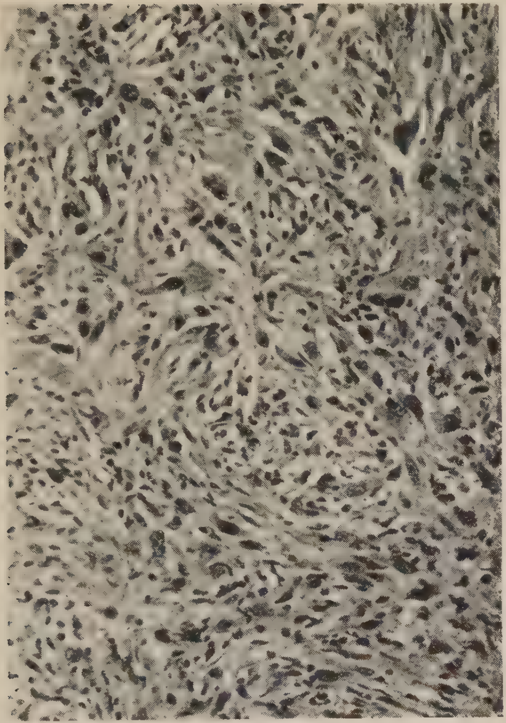


FIG. 1.
Photomicrograph of tumor occurring at injection site of sulfanilamide in lard in an albino rat.

numerous, there were occasional tumor giant cells and direct extension into adjacent tissue was obvious. Fig. 1 shows the microscopic appearance of the tumor. This tumor was transplanted to 14 rat littermates. Seven were fed a stock diet and 7 fed a diet containing 5% sulfanilamide. The controls grew faster than the test group and the tumors were smaller in the sulfanilamide group. However, 6 weeks after the transplant all animals in both groups were grossly deformed by tumors which were identical in structure and resembled closely the original tumor. There was no apparent difference in the 2 groups.

Thirteen months after the first injection a second rat in the group had developed a huge mass in the right groin. This was also a spindle cell sarcoma which was anaplastic, invasive and transplantable. No metastases occurred in this instance however.

The fate of the rest of the group is not as significant. Two died of lung abscesses at 8 months and 13 months after the first injection. The remainder were killed at 16 months after the first injection and no abnormalities noted at autopsy at this date.

Group II. Sulfapyridine injections (test animals). Injections exactly similar to Group I were made with 0.015 g of sulfapyridine in 40 rats. Three rats developed spindle cell sarcomata at the injection site. They were sacrificed at 16, 19, and 20 months. A considerable number of rats died of other causes during this period. Fifteen rats were still alive at 21 months. These were sacrificed and no tumors were found at autopsy.

Group III. Benzanthracene injections (positive controls). Ten rats were injected in an exactly identical manner as described in Group I using 0.015 g of 1:2:5:6 dibenzanthracene in 0.5 cc lard. Six of these animals developed sarcomata at the injection site. There were lung metastases in 2 of the animals. The tumors were all anaplastic, invasive and transplantable. The animals were killed when the tumor became obvious. The first animal to develop a tumor in this group was sacrificed at 6 months after the first injection, and the last occurred at 15 months. The remaining animals died of other causes.

Group IV. Lard injection (lard control). Thirty rats were injected with 0.5 cc of lard in the same manner as listed above. No deaths had occurred in this group at 16 months when the animals were sacrificed and autopsied. No abnormalities were found in this group.

Group V. No injections (negative controls). Thirty rats of the same strain were observed for a period of 16 months for the spontaneous development of tumors. All the rats were alive at the end of that period and were sacrificed. No tumors were noted at autopsy.

Discussion. Since the artificial production of tumors in animals is a resultant of numerous factors such as species, susceptibility, potency of the inducing agent and the age of the animal, it has always been difficult to obtain unequivocal results in evaluating any substance for possible carcinogenicity. The species variance to sulfanilamide is probably great and it would be fallacious to apply these meager data to higher species. Are the sul-

TABLE I.
Summary of Injection Experiments

Material inj.	Amt	Vehicle	No. of rats	Tumors	%
I. Sulfanilamide	30 mg	Lard	10	2	20
II. Sulfapyridine	30 ”	”	40 (18)*	3	7.5 (16⅔)*
III. Dibenzanthracene	30 ”	”	10	6	60
IV. Lard	0.5 cc		30	0	0
V. No injection			30	0	0

* Only 18 rats survived the entire experiment (see text).

fonamides carcinogenic for man? This question which occurred to the early workers remains as yet unanswered. Some analogy may perhaps be drawn from the experience with aniline dyes and carcinoma of the bladder in man. Hueper[4] has summarized the studies and experience in this field and it would seem that aniline dye workers develop bladder tumors in a statistically significant higher incidence than other people their age who are not exposed to these dyes. The average time between exposure and appearance of the tumor was 18 years. So many concomitant factors operate in such a period that conclusive evidence can be obtained only by statistical methods. However, if sulfonamides exert a similar influence and have the same latent period, it may be that an increase in the incidence of bladder tumors may be seen in or about 1955. Although highly speculative, this possibility deserves consideration because of obviously grave implications.

Conclusions. 1. Under the conditions listed it was observed that albino rats injected with sulfanilamide or sulfapyridine in lard developed sarcomata at the injection site. This incidence was less than the number of tumors occurring following the injection of dibenzanthracene under identical conditions. 2. The occurrence of tumors in Swiss albino mice following the injection of sulfanilamide in lard is reported in confirmation of previous work.

---

[4] Hueper, W. C., *J. Indust. Hyg.*, 1934, **16**, 255.

16476

Lipids of the Fasting Mouse. V. Liver Phospholipid, Lecithin and Cephalin.

H. C. HODGE, P. L. MACLACHLAN, W. R. BLOOR, E. WELCH, S. L. KORNBERG, AND M. FALKENHEIM.

From the Department of Biochemistry and Pharmacology, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y.

The young, adult mouse has been shown to mobilize available carcass fat in the first 2 days of a fasting period.[1] The liver total lipid increases during this period perhaps due to the failure of a phosphorylating mechanism to meet the emergency by converting the neutral fat to phospholipid prior to burning or transporting it. The incorporation of radio phosphorus (P32) into liver phospholipid metabolism is increased during fasting: this increase has been found in a series of experiments in which the total phospholipid, lecithin and cephalin fractions of the livers of mice fasted 5 days were studied.

Experimental. Three months old, male, albino mice were fasted as described previously.[1] Twenty-four hours before sacrifice, a small volume of a solution containing P32 as Na_2HPO_4 was injected intraperitoneally;

---

[1] Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Stoneburg, C. S., Oleson, M. C., and Whitehead, R., *J. Biol. Chem.*, 1941, **139**, 897.

radioactivity determinations were made with a Bale dipping type counting tube.[2] At the time of sacrifice, the livers were removed quickly and analyzed by standard procedures.[3]

Liver Phospholipid. The amount of phospholipid in the liver decreased approximately 43% during fasting; the normal liver contained about 36 mg; the liver after 5 days fasting contained about 20 mg of phospholipid. This loss of phospholipid was almost exactly parallel to the loss of liver weight, so that the concentration of phospholipid in the liver remained approximately constant; this was in complete agreement with previously-reported data.[1,4] The normal percentage of phospholipid was about 3.24 (moist weight basis); with fasting, the values for succeeding days ran 2.97, 3.35, 3.20, 3.35 and 3.46. The tendency of the phospholipid percentage to go through a minimum on the first fasting day has been observed before; however, no reason for such a decrease has been surmised.

It is noteworthy that the % P32 in the liver phospholipid increased as the amount of phospholipid decreased during fasting. The magnitude of the increase in % P32 dose was unexpectedly large; a 2-fold increase was observed on the second day and a 4-fold increase on the fifth day (Table I).

Lecithin and Cephalin. Determined separately, both the lecithin and the cephalin fractions of the liver phospholipid showed a significant decrease on the first day of fasting and again on the fourth and fifth days. This conformed with the observed decrease in the total liver phospholipid; in fact, the combined values of lecithin and cephalin equalled over 90% of the total phospholipid values taken day by day.

Lecithin normally achieves higher values of % P32 dose per mg than does cephalin; this ratio is of the order of 2. Riley reported a similar finding in normal young rats; lecithin phosphorus had a specific activity of 825-966,

TABLE I.

Data on Liver Lipids and % P_{32} Dose in the Liver Phospholipid of Normal and Fasting Mice.*

	Total lipid		Phospholipid				Lecithin				Cephalin			
			Total		% P_{32} Dose per mg		Total		% P_{32} Dose per mg		Total		% P_{32} Dose per mg	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Normal	77.0	14.2	35.6	5.7	.056	.012	19.8	4.2	.064	.014	15.0	1.5	.033	.010
Fasted 1 day	108.5	32.0	27.3	5.8	.088	.020	14.8	3.0	.108	.022	11.1	2.7	.059	.020
,, 2 days	139.4	18.9	28.0	2.4	.113	.025	15.1	2.1	.156	.028	12.2	1.4	.066	.020
,, 3 ,,	63.4	22.5	27.7	4.6	.136	.085	14.5	2.4	.097	.029	12.5	3.0	.050	.017
,, 4 ,,	43.7	12.4	23.3	5.1	.086	.036	11.9	3.8	.114	.052	9.1	2.3	.053	.039
,, 5 ,,	38.0	4.9	20.0	1.7	.188	.070	10.6	1.0	.215	.121	8.3	1.1	.138	.055

* The numbers of mice in each group are given in Table II of "Lipids of the Fasting Mouse. IV. Liver Total Lipid Content." PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 137-139.

---

[2] Bale, W. F., Haven, F. L., and LeFevre, M., *Rev. Scient. Instruments,* 1939, **10**, 193.

[3] Welch, E. A., *J. Biol. Chem.,* 1945, **161**, 65.

[4] MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. B., *J. Biol. Chem.,* 1942, **143**, 473.

whereas cephalin phosphorus from the same livers averaged 363-735.[5]

The changes in the % P32 dose per mg of liver lecithin and cephalin paralleled each other. The values increased during the first 2 days of fasting, fell on the third day, and increased again markedly on the fifth day. With the exception of the value obtained on the third day of fasting, the % P32 dose per mg total liver phospholipid represented approximately the average of the values for the % P32 dose per mg lecithin and cephalin.

During the first 2 days of fasting when depot fat was being rapidly mobilized, the increased phospholipid metabolism in the liver was evidenced by an increase in the % P32 dose per mg liver phospholipid. As previously observed[6] the liver total lipid reached a maximum on the second day of fasting. In some respects a similar condition obtains on a low choline diet, *viz.*, the liver neutral fat is increased and the lecithin level falls below normal; the administration of choline reverses this relationship.[7]

The accumulation of fat on the second day of fasting might account for the decrease in the % P32 dose in the liver lecithin and cephalin on the third day. Later, as the fat moved out of the liver (third to fifth day of fasting) the phospholipid metabolism presumably increased and, by the fifth day, the value for the % P32 dose in the liver phospholipids attained very high values (4 times normal). In the light of recent transmethylation studies it seems reasonable to suppose that more of the choline precursors were made available when protein metabolism was accelerated during the last days of fasting.

Summary. Young, adult, male albino mice lost almost half the liver total phospholipid during a 5-day fast. The liver weight also decreased in such a fashion that the phospholipid concentration remained approximately constant. The amounts of lecithin and cephalin decreased in proportion. The % P32 dose per mg of phospholipid increased markedly during fasting and, although the % P32 dose per mg lecithin is normally higher than that of cephalin, during fasting the values increased markedly for both fractions.

---

[5] Riley, R. F., *J. Biol. Chem.*, 1944, **153**, 535.

[6] Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Welch, E., Kornberg, S. L., and Falkenheim, M., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 137.

[7] Fishman, W. H., and Artom, C., *Federation Proc.*, 1945, **4**, 90.

16477

Effect of Asphyxia on Blood Coagulation in Dogs.

JOSEPH L. PONKA AND CONRAD R. LAM. (Introduced by Oliver H. Gaebler.)
From the Division of General Surgery, Henry Ford Hospital, Detroit.

Several manuals on postmortem examinations contain statements to the effect that a fluid state of the blood in a body is suggestive of oxygen deficiency at the time of death. For example, in describing the conditions found after death from asphyxia, Ross[1] stated "The blood is fluid and very dark in color owing to its being chemically almost completely reduced." Under the caption "Drowning," the same author said "The blood on the right side is dark in color and fluid (asphyxia)." In the section on asphyxia, Warthin's "Practical Pathology"[2] contains the following: "The lips, skin of the face and neck, and the finger nails may be deeply cyanotic; the blood is dark and fluid."

This experimental study was undertaken to determine the effect of severe asphyxia on the clotting mechanism as judged by *in vitro* tests on blood withdrawn from living animals

---

[1] Ross, J. M., *Post-mortem Appearances*, New York and London, Oxford Univ. Press, 1939.

[2] Warthin, A. S., *Practical Pathology*, Ann Arbor, George Wehr, 1928.

TABLE I.
Results of Coagulation Time Determinations in First Series.

Exp. No.	Blood coagulation time (min.) Normal	During asphyxia	Recovery	Period of asphyxia (min.)
1	8	6	7	5
2	7	7	7	7
3	7	9	7	5
4	6	5	5	5
5	7	6	7	6
6	6	5	5	6
7	9	9	8	5
8	7	6	5	7
9	6	6	*	5
10	7	7	10	6
11	10	9	*	5

* Expired.

and by observations of the postmortem state of the blood in animals killed by asphyxiation.

Method. A common method for determining the coagulation time of the blood is that of Lee and White.[3] A sample of venous blood is drawn and placed in 3 test tubes. One of these is inverted at 30-second intervals until a clot has formed which retains its position in the bottom of the tube. The second and then the third tubes are treated in a similar manner. The time from the drawing of the blood until the clot forms in the third tube is taken as the coagulation time. In this investigation, it was necessary to prevent contact between the blood sample and the oxygen of the air. We tried putting the blood under a layer of mineral oil in the test tube, but the greasy inner wall of the tube and the slight emulsification caused by the repeated inversions of the tube made it difficult to judge the end-point. A more satisfactory method involved the use of rubber capped tubes filled with the inert gas helium. The blood sample was placed in the tube by piercing the diaphragm of the cap with the needle on the syringe.

The first series of experiments was carried out on 11 healthy dogs. The animals were rendered unconscious with ether, after which a catheter with a distensible balloon was inserted into the trachea. Ether-oxygen anesthesia was continued through this closed system. Both femoral veins were exposed through surgical incisions to permit quick and accurate vein punctures. A sample of blood was removed from the right femoral vein and divided into 3 helium tubes. The clotting time of this sample was noted. The endotracheal catheter was then clamped for about 5 minutes or until the animal was almost dead of asphyxia. At this point, the tongue would be purplish black in color, the heart action feeble and the respiratory movements spasmodic or absent. A second sample of blood was removed from the left femoral vein and its clotting time determined. With 2 exceptions, the prompt administration of oxygen by artificial respiration with the anesthesia apparatus permitted the animals to recover, after which a sample of the re-oxygenated blood was obtained.

In the second series of experiments, 3 anesthetized dogs were killed by asphyxiation, the endotracheal tube being clamped until there was no sign of life. Autopsy was performed after 2 hours and the condition of the blood in the heart and great vessels was noted. A fourth animal was killed instantly by electrocution, one electrode being placed over the heart (non-asphyxial death).

Results. The results of the 11 experiments in the first series are shown in Table I. It will be noted that there were no significant differences in the clotting times of the specimens removed before, during and after the periods of severe asphyxia. There was certainly no tendency for the second specimen, obtained during asphyxia, to show a prolongation of clotting time which would have indicated a breakdown of the coagulation mechanism.

---

[3] Lee, R. I., and White, P. D., *Am. J. Med. Sc.*, 1913, **45**, 495.

Postmortem examination of the animals killed by asphyxia revealed firm clots in the heart and great vessels. The blood of the animal killed by electrocution was also coagulated.

Conclusion. It could not be demonstrated that severe asphyxia has any effect on the clotting properties of the blood of the dog.

16478

A Concomitant Change in Mitochondria and Virulence of a Transplanted Lymphoid Leukemia.

RICHARD A. MILLER* AND MARTHA J. TAYLOR.

From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N.Y.

Potter and Ward[1] published the numbers of mitochondria in lymphocytes from the blood of C58 mice inoculated with several of the transplantable leukemias which had originated from different spontaneous cases in this laboratory. Two highly virulent leukemias, Lines I and MLiv which had then been transferred 800 or more times, killed the mice 4 days after inoculation with a standard number of cells. Two relatively less virulent leukemias, Line S transferred about 90 times and Line U transferred 2-9 times, killed the mice 7 and 15 days after inoculation.

Since that study was made Line I and Line MLiv have passed through about 500 additional transfers and the interval before death is still about 4 days.[2] In Line U and Line S, however, during about 350 additional transfers, the interval between inoculation and death has decreased to about 4 days. The present study was undertaken to determine what changes if any had occurred in the mitochondria of these leukemias. Lines U and I were selected as representative of leukemia which in one instance had greatly increased in virulence, and in the other had shown no change in the interval between inoculation (with a standard number of cells) and death, since the previous study.

Materials and methods. Virtually the same procedures were used as in the earlier study.[1] Blood from the tails of C58 mice (4 to 6 weeks old) inoculated with either Line I or Line U was studied during the terminal stages of the disease when the number of leucocytes in the peripheral blood was great. Control data were provided by counts of mitochondria in animals just before inoculation with leukemia as well as in their untreated littermates. Mitochondria stained with Janus green were counted in 500 to 1000 lymphocytes (50 or 100 cells selected at random from each mouse) from each of the leukemic groups and from control animals. Slides were prepared by flooding them with a solution of Janus green and neutral red in absolute alcohol, after which they were drained and allowed to dry dust free. Each 10 cc of this solution contained 14 drops of 1% neutral red in absolute alcohol and 27 drops of 1% Janus green in absolute alcohol. A drop of blood was placed upon such a slide, covered with a glass slip and sealed about the edges with vaseline.

The diameter of lymphocytes was measured with a calibrated ocular micrometer in blood smears stained with Wright's solution. If the lymphocytes were oval rather than round the diameter was assumed to be the average of the length and breadth.

Observations. Table I presents the mean

---

* Present address: Department of Anatomy, University of Minnesota, Minneapolis, Minn.

[1] Potter, J. S., and Ward, E. N., *Cancer Research*, 1942, **2**, 655.

[2] MacDowell, E. C., *Cold Spring Harbor Symp. Quant. Biol.*, 1946, **11**, 156.

TABLE I.
Average Number of Mitochondria in Lymphocytes of Normal C58 Mice and Mice Inoculated with Line I and Line U Leukemia.

	No. of transfer	Leucocytes per cu mm	No. of lymphocytes counted	Avg No. of mitochondria	Standard deviation	Range
Normal		17,309	550	32.55 ± .55	12.85 ± .39	8- 89
Line I	1510-1513	147,476	500	43.35 ± .62	13.95 ± .44	8-115
” U	357-370	101,180	1,000	73.16 ± .70	22.00 ± .49	6-143
Normal*			1,000	18.77 ± .20	6.40 ± .14	5- 42
Line I*	1017-1046		1,000	32.07 ± .27	8.55 ± .19	12- 60
” U*	1-9		1,000	34.42 ± .31	10.04 ± .22	6- 67

* Data of Potter and Ward, '42.

number of mitochondria for the three groups of C58 mice. Normal mice have the fewest mitochondria; in mice inoculated with Line I the number is increased by approximately a third; in mice inoculated with Line U the number is more than doubled. The modes and upper limits of the 3 frequency distributions (Fig. 1) differ in the order of the means, but the lower limits coincide.

Data from the earlier study of Potter and Ward[1] are presented for comparison (Fig. 1, Table I). Although the relative position of normal, Line I, and Line U with respect to the number of mitochondria in lymphocytes is the same, the present counts are consistently greater than the previous counts. This appears in the means, modes and upper limits of the frequency distributions. The total range for each of the two leukemic groups is now more than double the previous ranges but the lower limits are still nearly the same as before.

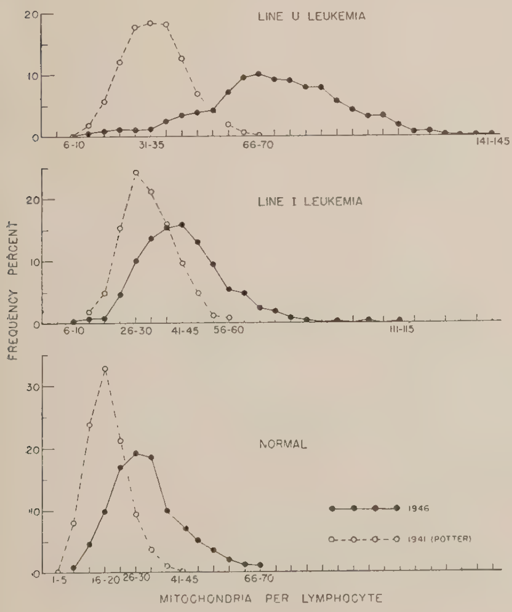


FIG. 1.

An explanation for the discrepancy in the number of mitochondria in normal lymphocytes could not be established. Theoretically there are two possibilities. Either there has been an actual change in the number of mitochondria or an apparent one resulting from differences in technic. The fact that C58 has long been a highly inbred strain argues against the first possibility. Although the procedure of Potter and Ward was followed as closely as possible, with one known exception, unrecognized differences in technic may have affected the results. The present counts were made on slides coated with a stronger solution of dyes since, in a few preliminary counts on slides prepared according to their method, the mitochondria faded so rapidly that only a few cells could be studied. Although these preliminary counts average slightly lower than later ones, they were higher than the 1941 counts, and it is felt that the difference in the preparation of the slides cannot account for the discrepancy.

Whatever may be the explanation of the present greater number of mitochondria in normal lymphocytes, the difference in the number of mitochondria between the 2 counts of Line U is greater than the difference between the 2 normal counts. The average number of mitochondria in normal lymphocytes is now 73% greater than previously, whereas the mean of Line U is now 113%

TABLE II.
Average Diameter of Lymphocytes of Normal C58 and Mice Inoculated with Line I and Line U Leukemia.

	No. measured	Avg diameter, microns	Standard deviation	Range, microns
Normal	550	9.87 ± .06	1.49 ± .04	6-15
Line I	500	12.85 ± .09	1.96 ± .06	7-18
” U	1,000	12.17 ± .07	2.24 ± .05	7-18

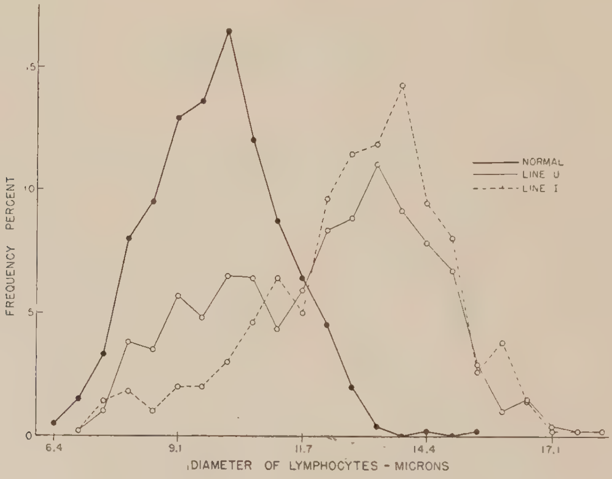


Fig. 2.

larger than the previous count. This notable increase in the number of mitochondria of Line U is most readily appreciated by comparing the 2 frequency distribution curves of the 2 normal counts with the 2 counts of Line U (Fig. 1). While the 2 normal curves overlap in large measure, the area of overlapping of the 2 Line U curves is small. In contrast, comparison of the Line I curves with the normal curves does not reveal an increase in the number of mitochondria in Line I which exceeds the increase between the 2 counts in control mice.

No change in the morphology of the mitochondria could be detected in a comparison with the descriptions and photographs of the earlier study. Now, as in the past, most of the mitochondria of normal lymphocytes are small distinct granules but there are some rods, filaments and indistinct granules. In Line U granules also predominate but have a greater range of size than those of normal lymphocytes. Rods, filaments and indistinct small granules are also seen. As in the previous study, the proportions of the several shapes and sizes varied from animal to animal inoculated with Line U. Mitochondria in lymphocytes of Line I have maintained the characteristic morphology previously described. The large granules of swollen appearance and the large and small discrete granules are so distinctive that they easily identify lymphocytes of this line.

Lymphocytes of the 3 groups also differ from each other in mean cell diameter (Table II). The range of cell size in the leukemic groups is greater than in the control group but the lower limits are almost the same. In range, the 2 leukemic groups are exactly the same but Line U has a greater proportion of the smaller lymphocytes (Fig. 2). Similarly, in the previous study the average diameter of lymphocytes of Line U was smaller than that of Line I.

Summary and conclusions. New data presented in conjunction with data published by Potter and Ward indicate that a marked increase in the number of mitochondria in lymphocytes of C58 mice inoculated with Line U leukemia has occurred in the course of 350 transfers. This increase in the number of mitochondria has occurred concomitantly with a decrease in the killing time, following a standard number of cells, from about 15 days to 4 days. The number of mitochondria in lymphocytes of Line I has not shown a similar increase in the course of 500 transfers made during this same period of time, and the interval between inoculation and death has remained unchanged at approximately 4 days. In making these new counts it was not possible to follow in every respect the technic for staining of mitochondria used by Potter and Ward.

Certain other differences between Line U and Line I have not changed since the time of the previous study of these leukemias. In spite of the increase in mitochondria, the lymphocytes of Line U remain smaller than those of Line I. In morphology, the mitochondria have retained the characteristics which distinguished them previously. Since the range in size of lymphocytes is the same in the two leukemias, although the means are different, if size is inversely correlated with the age of the lymphocyte as has been suggested,[3,4] then the 2 groups of lymphocytes share the same ages and stages of differentiation. The persisting differences between Line U and Line I, therefore, may be characteristics of the lines of leukemia and not differences due to the age and developmental stage of the lymphocytes.

---

[3] Wiseman, B. K., *J. Exp. Med.*, 1931, **54**, 271.

[4] Potter, J. S., and Richter, M. N., *Arch. Path.*, 1933, **15**, 198.

16479

Distribution and Rate of Disposition of Myanesin in Dogs.

JAMES L. MORRISON, PASSIE SAPERSTEIN, HARRY A. WALKER, AND ARTHUR P. RICHARDSON.

From the Department of Pharmacology, Emory University School of Medicine, Emory University, Ga.

One of the most interesting characteristics of the action of myanesin* (3-(orthotoloxyl)-1,2-propanediol) is its shortness of action.[1-3] By the use of 3 different analytical methods the duration of pharmacological effect has been correlated with concentration of free myanesin in plasma.[3,4] It has also been demonstrated that less than 1% of myanesin administered intravenously can be recovered in urine as free myanesin. After hydrolysis with acid, material has been obtained from the urine which may be nitrated or coupled with diazotized dinitro-aniline to an extent which accounts for at least 30 to 40% of the total myanesin administered. It would appear, therefore, that myanesin is rapidly degraded in the body and that its short action is due almost entirely to rapid metabolic change. If myanesin is to be used intelligently, more information is needed as to the character of its absorption, excretion, distribution, and disposition. This report summarizes certain aspects of this problem.

Methods. Of the 3 methods proposed for the determination of myanesin in body fluids and tissues,[3,4] the coupling reaction with diazotized dinitro-aniline described by Titus, Ulick and Richardson is most suitable since

---

* We wish to thank Squibb Institute for Medical Research for the sample of myanesin used in these studies.

[1] Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, **1**, 265.

[2] Burke, J. C., and Linegar, C., to be published.

[3] Titus, E. O., Ulick, S., and Richardson, A. P., *J. Pharm. and Exp. Ther.*, to be published.

[4] Wyngaarden, J. B., Woods, L. A., and Seevers, M. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 256.

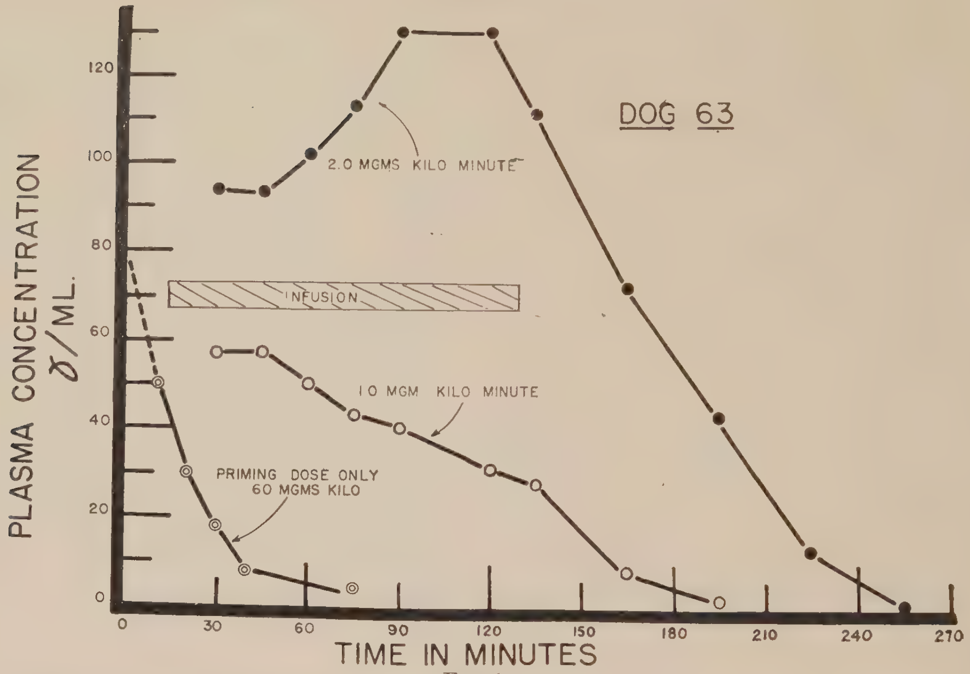


FIG. 1.

A typical experiment illustrating the effect of rate of infusion on plasma concentration of myanesin following a priming dose of 60 mg per kilo. Drug administered intravenously at the rate of 1 ml per minute and concentration adjusted to give the appropriate dose of drug.

it is both sensitive and the analysis is not complicated by high tissue or reagent blanks. The results reported here have all been obtained with this method.

For the determination of "total" myanesin, samples were acidified with an equal volume of concentrated hydrochloric acid; heated for 10 minutes; extracted into chloroform; evaporated; residue taken up in 85% phosphoric acid; and coupled as in the method for determination of free myanesin. In the absence of definitive information as to the nature of the degradation product it was not possible to say exactly what our recoveries were for "total" myanesin, nor can one be certain that this is the best way in which the analysis could be carried out. However, since it was possible to account for 30-40% of the compound administered, the method described must be at least an approximation of "total" myanesin.

Rate of disposition of myanesin. The general plan of these experiments is illustrated by results presented in Fig. 1 for a single dog. Plasma concentrations were determined at intervals following the intravenous injection of a single dose of 60 mg per kg, until all drug had disappeared from the plasma. Several days later a glass cannula was inserted into a femoral vein under local anesthesia; a priming dose of 60 mg per kg of myanesin was injected intravenously and 5 minutes later a continuous infusion of drug into the cannulated vein was begun. Rate of infusion was kept constant at 1 ml per minute and concentration of drug adjusted so as to give each animal either 1 or 2 mg per kg per minute of infusion. Infusion was continued for a period of 2 hours. Blood samples for determination of plasma concentration were taken at half hour intervals during the period of infusion, and at hourly intervals thereafter until plasma concentrations had fallen to zero.

Rate of disposition of myanesin following infusion of both 1 and 2 mg per kg per minute was determined in 6 dogs. Ten other dogs were infused only at a rate of 2 mg per kg per minute.

Fig. 2 summarizes all experiments in 16 dogs carried out as described above. For pur-

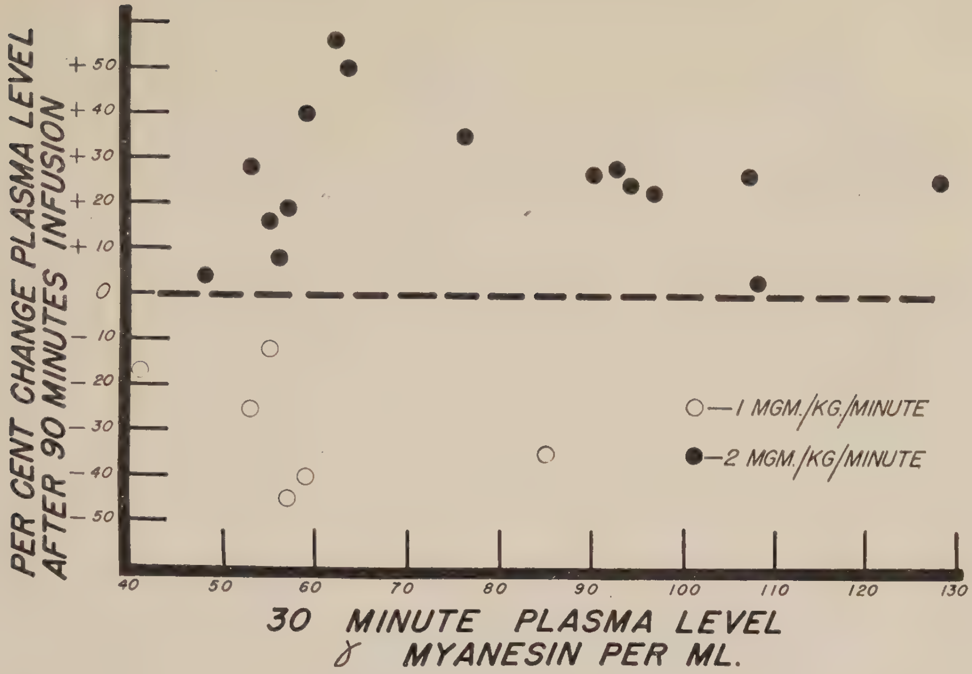


FIG. 2.

Summary of experiments showing relationship of rate of infusion to change in plasma concentration. Each point represents per cent change in concentration from thirty minutes after priming dose as compared to plasma concentration 90 minutes later.

poses of comparison it was assumed that 30 minutes after the priming dose (and 25 minutes after starting the infusion) was a sufficiently long period of time to allow for a balance to be established between the processes of distribution, degradation, and excretion. Change in plasma concentration beyond this point compared to rate of infusion should give an estimate of the ability of each animal to dispose of the drug. It is readily seen from Figs. 1 and 2 that infusion of 2 mg per kg per minute resulted in an increasing plasma concentration and therefore is greater than the rate of disposition. On the other hand infusion of 1 mg per kg per minute was insufficient to maintain plasma levels, thus one can conclude that under the conditions described dogs can dispose of between 1 and 2 mg per kg per minute.

Plotting the results as shown in Fig. 2 also illustrates another point, namely, that between plasma levels of 50 and 130 μg per ml the rate of disposition can not be correlated with plasma concentration.

That disposal of myanesin is due to metabolic change rather than excretion was suspected from earlier experiments.[1,3,4] Additional evidence for this point of view was obtained from some of the present continuous infusion experiments in which urine was collected during the infusion period and analyzed for free myanesin. In the several experiments in which this was done less than 1% of the drug infused could be found in the urine. Since a major portion of myanesin does eventually appear in the urine, in combined form, it is obvious the rapid disposal of the drug must be due to metabolic alteration.

Distribution in body fluids and tissues. Preliminary calculations of the apparent volume of distribution of myanesin following a single intravenous injection[3] gave a value of approximately 70% of body weight. It was to be expected therefore that the drug was widely distributed possibly in total body water. In order to study this more exactly 7 unanesthetized dogs were infused intravenously with myanesin at the rate of 2 mg per kg per minute as described above. After 2 hours infusion, blood, saliva, and spinal fluid samples were

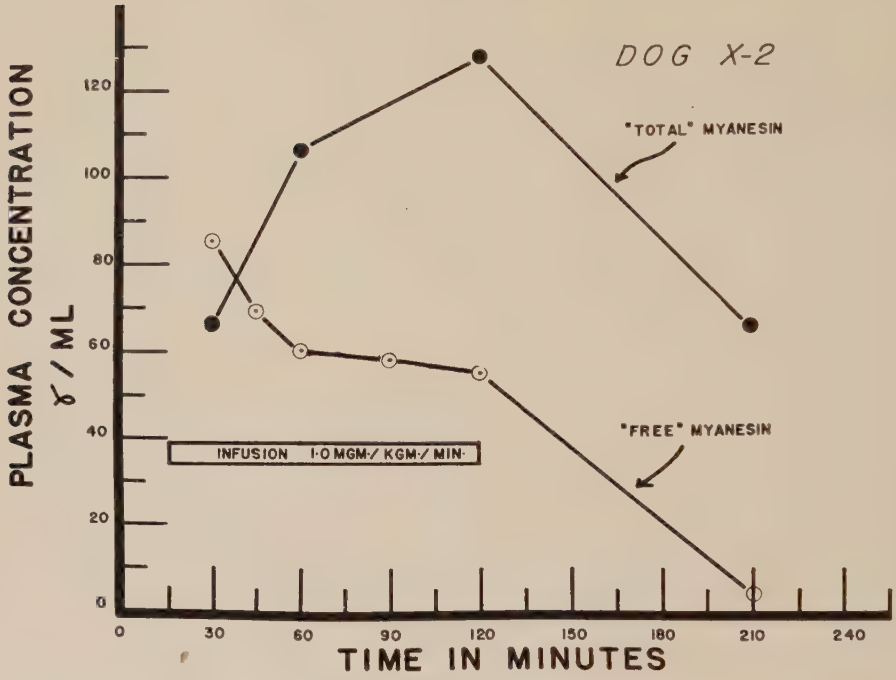


FIG. 3.

Typical experiment illustrating relationship of free and "total" myanesin in plasma following intravenous infusion. "Total" drug determined after hydrolysin with hydrochloric acid.

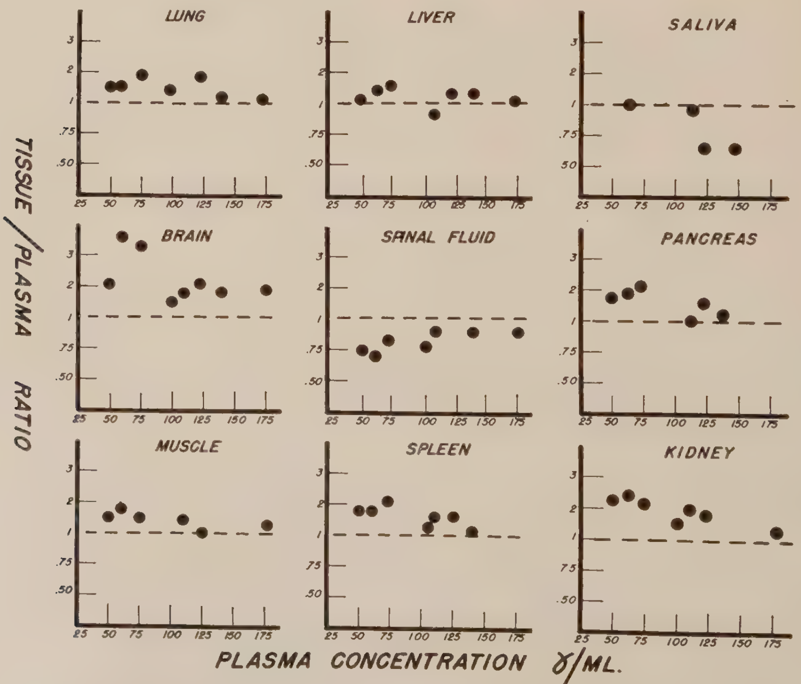


FIG. 4.

Tissue/plasma concentration ratio after continuous infusion of myanesin for 2 hours. All determinations are of free drug.

taken and animal killed by the intravenous injection of air. Tissue samples were removed as rapidly as possible and prepared immediately for analysis. Each sample of tissue was weighed; ground in a Waring blendor to form a coarse suspension and finally ground with a Ten Broeck tissue grinder until each sample could be handled with a tuberculin syringe. In order to facilitate grinding each sample of tissue was mixed with an equal volume of saline, except for muscle with which 2 volumes of saline were used for each volume of tissue. Preliminary experiments indicated that added myanesin could be recovered satisfactorily from such tissue mashes. However, it was not possible to analyze for "total" myanesin since tissue-reagent blanks were excessively high.

The results of all experiments are summarized in Fig. 4 in which ratios of tissue/plasma concentration are plotted against plasma concentration. As predicted it is obvious that myanesin is widely distributed, and approximately according to water content of each tissue. A notable exception to this, however, is seen in the case of brain in which the ratio of tissue/plasma was always more than unity and average approximately 2. The explanation for this finding is at present obscure, but of considerable interest since the outstanding actions of myanesin are on the central nervous system. Since spinal fluid and saliva concentration were consistently lower than plasma, one wonders if perhaps distribution of myanesin may not in part be conditioned by lipoid solubility as well as water solubility.

Discussion. The above experiments furnish concrete evidence that the brief action of myanesin is due primarily to rapid metabolic alteration. Since myanesin has been suggested for use in a number of chronic diseases[5,6] where prolonged action is desirable, it is important that more data concerning the characteristics of the processes involved in the alteration of the free drug should be obtained. Such information may furnish important leads as to means whereby longer acting drugs may be synthesized.

Summary. 1. From a study of plasma concentrations of animals infused continuously, it has been shown that dogs can dispose of between 1 and 2 mg of myanesin per kg per minute, irrespective of plasma concentration between the limits of 50 and 130 μg per ml. 2. The rapid disposition of myanesin is due primarily to metabolic alteration. 3. Myanesin is widely distributed in the body approximately according to water content. A notable exception to this is found in the brain where tissue/plasma ratios of greater than 2 are observed.

---

[5] Stephen, C. R., and Chandy, J., *Canad. Med. J.*, 1947, **57**, 463.

[6] Schlesinger, E. B., Drew, A. L., and Wood, B., *Am. J. Med.*, 1948, **4**, 365.

16480

Effect of Cycloheptenylethyl Barbituric Acid, "Medomin," on Intestinal and Uterine Smooth Muscle.*

William A. Halbeisen, Charles M. Gruber, Jr., and Charles M. Gruber.

From the Departments of Pharmacology and Internal Medicine, Jefferson Medical College, Philadelphia, Pa.

The following investigation was undertaken to determine the effect of cycloheptenylethyl barbituric acid "Medomin" on intestinal and uterine smooth muscle and the effects of a number of short and ultra-short barbiturates on the intact intestine of the non-anesthetized dog.

Methods. Excised Intestine. Excised longi-

---

* This work was made possible through a grant by the Geigy Co., Inc., for research in the sciences.

tudinal segments of rabbit intestine were used. The animals were killed by cerebral concussion and exsanguination. Some of the tissues were used immediately after being removed while others were kept in a refrigerator in Locke's solution until needed.

One end of the muscle was fastened to an L-shaped glass rod and the other end to a muscle lever by means of ligatures. The muscles thus suspended, together with the L-shaped glass rod, were placed in a pyrex glass cylinder containing freshly made Tyrode's solution with a pH of 7.8. Two muscle segments, one of the duodenum and one of the ileum, were suspended in the same bath. The temperature of the bath was maintained at 38.5°C ± 0.2°C.

Cycloheptenylethyl barbituric acid, phenobarbital sodium and seconal sodium were used in this investigation. The drugs were weighed and placed in volumetric flasks and Tyrode's solution added to volume. In the case of cycloheptenylethyl barbiturate a solution of sodium hydroxide was added to form the soluble sodium salt before the addition of Tyrode's solution. A given amount of these solutions was added to the bath to make dilutions ranging from M/20,000 to M/1,000.

Intact Intestine. Trained non-anesthetized dogs with Thiry-Vella loops of the small intestine were used in studying the effects of this and a number of barbiturates on the intact intestine. The method of preparation of the animals was the same as that previously described by one of us.[1] The changes in the general tonus and in the rhythmical contractions were recorded by means of a rubber balloon placed within the lumen of the gut.[1] The balloon was connected to a modified Brodie bellows. The pressure within the balloon was 15 cm of water. After a normal control record had been secured the drug was injected intravenously.

Excised Uterus. Uterine segments taken from rabbits and cats were used for this research. The method of preparation of the segments was like that described for excised intestine. The same apparatus was employed for maintaining a constant temperature of the bath, for oxygenating and for recording the contractions of the uterus. Locke's solution having a pH 7.6 was used as the bath. Two segments, one from each horn of the uterus was always used simultaneously and suspended in a manner similar to that employed in studying excised segments of intestine. The drugs were weighed and dissolved in Locke's solution just preceding the experiment. A given amount of this solution was added to the bath to make dilutions of M/15,000 to M/1,000.

Intact Uterus. The method employed in investigating the intact uterus of rabbit, cat or dog was similar to that used by Barbour.[2] The animal was anesthetized with ether by inhalation. A cannula was placed in the trachea and the ether continued. Artificial respiration was used to rule out the possible effect of anoxia, in case the respiratory center was excessively depressed by the barbiturate. The uterine horns were exposed by a midline abdominal incision. A glass tube, such as described by Barbour,[2] was passed into the abdomen over the uterus through the wound and the abdominal muscles and skin securely fastened around the tube making the joint water tight. The glass tube was kept partly filled with warm Ringer's solution. The uterine horns with their circulation and nerve supply intact were brought up into the glass tube and by means of linen threads fastened to muscle levers, which wrote on smoked paper on a kymograph drum.

Results. Excised Intestine. Twenty-eight, 26 and 30 experiments were performed with cycloheptenylethyl barbiturate, seconal sodium and phenobarbital sodium respectively upon 10 pairs of excised segments of rabbit intestine. A decrease in the general tonus and in the height of the rhythmical contractions were recorded in every instance. A comparison of the results of the 3 barbiturates used in these experiments revealed the fact that seconal is approximately twice as depressant as cycloheptenylethyl barbiturate, which in turn is twice as active as phenobarbital on the excised intestine.

---

[1] Gruber, C. M., Ellis, F. W., and Freedman, G., *J. Pharm. and Exp. Therap.*, 1944, **81**, 261; Gruber, C. M., *et al.*, *ibid.*, 1931, **42**, 27; Gruber, C. M., and Brundage, J. T., *ibid.*, 1935, **53**, 120; Gruber, C. M., *ibid.*, 1936, **56**, 432.

[2] Barbour, H. G., *J. Pharm. and Exp. Therap.*, 1915, **7**, 547.

Intact Intestine. Nineteen dogs with Thiry-Vella loops were used. Thiopental sodium (pentothal) (15, 20, 25 mg/kg) was injected 34 times in 14 animals; pentobarbital sodium (20 and 25 mg/kg) 6 times in 6 animals; thioethamyl (10, 20, 30 and 40 mg/kg) 14 times in 13 animals; hexobarbital soluble (evipal sodium) (25 and 40 mg/kg) 7 times in 7 animals; amytal sodium (15 mg/kg) twice in 2 animals; seconal sodium (15 mg/kg) twice in 2 animals, and cycloheptenylethyl barbiturate (10 to 25 mg/kg) 14 times in 4 animals. In every instance a sudden decrease in the general tonus was noted. In only 3 out of 79 injections of these barbiturates were we able to see a possible increase in the general tonus of the intact gut following the primary depression. In 48 of the experiments while these trained animals were recovering from the hypnosis of the drug they became so excited that the experiments had to be interrupted. Our results on these 19 animals following 79 injections of short and intermediate acting barbiturates fail to support Burnstein's[3] findings that the relaxation of the gut is followed in "every instance"—"by a gradual rise in the general tonus to above normal and increased rhythmic intestinal contractions which were maintained until the animal awakened." Our results are, however, in accord with those of Oettel,[4] who reports excitement to such extent in the animal coming out of the barbiturate (evipan and pernocton) hypnosis that the experiments had to be discontinued.

Cycloheptenylethyl barbiturate, like other barbiturates, causes a decrease in the general tonus and in the height of the rhythmical contraction of the intact gut in the unanesthetized dog. Both contractions and tonus returning to the control level in 30 to 60 minutes after the injection.

Excised Uterus. Fourteen experiments were performed on 10 pairs of segments of excised rabbit uterus and one experiment on a cat uterus. In all of these a prompt decrease in the general tonus and in the height of the contractions was observed. In a few segments the rate of the rhythmical contractions was increased by the drug.

Intact Uterus. Eleven experiments were performed on 7 rabbits, 21 experiments on 9 cats, and 2 experiments on 1 dog with the use of cycloheptenylethyl barbiturate. In cats pentobarbital sodium and seconal sodium were injected intravenously 4 and 6 times respectively. In the rabbit and dog, only a decrease in the general tonus and in the height of the rhythmical contractions were noted. In the cats, however, an increase in the general tonus was observed following 8 of the 21 intravenous injections of cycloheptenylethyl barbiturate, in 2 of the 4 injections of pentobarbital sodium and in 1 of the 6 injections of seconal sodium.

Summary. 1. Cycloheptenylethyl barbituric acid produces relaxation of the excised segments of intestine and uterus of cats and rabbits. 2. The intact intestine of dogs and the intact uterus of rabbits and dogs respond to the drug by a decrease in the tonus and a lessening of the rhythmic contractions. In the cats, however, increased tonus of the uterus was observed on some occasions. 3. In only 3 out of 79 injections of ultra-short, short and intermediate acting barbiturates was there a suggestion of an increase in the general tonus of the intact gut following the primary depression. About 50% of our experiments had to be discontinued during the period of intestinal relaxation because of the excitement produced in the animal as it was recovering from the barbiturate hypnosis.

---

[3] Burnstein, C. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 122.

[4] Oettel, H., *Arch. f. Exp. Path. u. Pharmakol.*, 1935, **177**, 323.

16481

Enzymatic Liberation of Inorganic Phosphate from Adenosinetriphosphate by Tissues of *Molgula manhattensis.*

Heinz Herrmann and Willard Hartman. (Introduced by J. S. Nicholas.)

From the Osborn Zoological Laboratory and Bingham Oceanographic Laboratory, Yale University, New Haven, Conn.

One of the important sources of chemical energy for various forms of work in cells and tissues is the enzymatic liberation of inorganic phosphate from adenosinetriphosphate (ATP).[1] The enzymatic systems which effect the liberation of the first two phosphate groups from ATP have been designated as apyrase.[2] A better understanding of the functional role of this important enzyme system may be possible through a survey of its comparative biochemistry. The following observations on the apyrase activity in the tissues of the ascidian, *Molgula manhattensis* (DeKay), are here reported as part of a contribution to such a survey.

Material and methods. The ascidians were collected during the fall at Milford (Conn.) harbor where they are common fouling organisms on wharf piles from mean low water to a depth of about 2 meters. The animals were transported to New Haven where the experiments were performed. Their physiological condition was checked before use by noting the contraction of the inhalent and exhalent siphons and the flow of blood through the heart.

After removal of the tough external wall of the tunic the internal organs were separated into the following fractions for analysis: (1) gonads (ovary and testis), (2) excretory organ, (3) pharynx, (4) stomach and intestine. In later experiments the digestive tract was divided into 3 parts: (1) the anterior folded portion or stomach, (2) the first half of the more tubular and less folded portion of the intestine (called henceforth the "midgut"), (3) the posterior half of the intestine, designated as "hindgut." In several experiments the entire tubular section was used removing the "stomach" but without separation into "mid" and "hindgut." In these instances the tissue used was simply designated as "gut."

After dissection and pooling the organs from at least 3-5 animals, 250 mg of the tissue (in each case) was homogenized[3] for 1 to 2 minutes with 5 ml of chilled distilled water. The homogenate was poured into 20 ml of chilled distilled water and portions of 0.1-0.3 ml of this suspension were used for determination of enzymatic activity according to the method of Dubois and Potter.[4] For the dry weight determinations the entire remaining quantity of suspension was precipitated by 10% trichloroacetic acid; the precipitate was washed in the centrifuge with 95% alcohol and dried overnight at about 100°C. Our substrates were obtained from commercial sources. ATP was supplied by Armour and Co. as the dibarium salt which contained 8.9% total N, 11.9% total P and 63% hydrolyzable P. The slight excess of total P (Theor. 10.9%) could be accounted for almost entirely by inorganic phosphate. Small volumes of solutions of the sodium salt were prepared by adding an equivalent amount of solid Na_2SO_4 to a solution of the barium salt in HCl. After removal of the barium sulfate the clear solution was neutralized with a small amount of 2N NaOH. Hexosediphosphate

---

[1] Potter, V. R., *Adv. Enzymology*, 1944, **4**, 201; Engelhardt, V. A., *Adv. Enzymology*, 1946, **6**, 147; Szent-Györgyi, A., *Chemistry of Muscular Contraction*, 1947, New York, 1-150; Sandow, A., *et al.*, *Ann. New York Acad. Sci.*, 1947, **47**, 665.

[2] Meyerhof, O., *J. Biol. Chem.*, 1945, **157**, 105.

[3] Potter, V. R., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, 1945, Minneapolis, 92-98.

[4] Dubois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

TABLE I.
Liberation of Inorganic Phosphate from Adenosinetriphosphate and Other Phosphate Esters by Homogenates of the Tissues of *Molgula manhattensis.*

The results are given as micrograms of P liberated per minute per mg dry weight during an incubation period of 15 min. at 38°C at a pH of 7.5 (if not indicated otherwise). Each figure gives the value obtained with a single sample of pooled tissue.

Tissue	Substrate	μg P
Pharynx	ATP	0.8
Excretory Organ	"	0.4
Gonads	"	0.7
Total Intestine	"	11.3
"Stomach"	"	3.8
Midgut	"	18.5
Hindgut	"	16.0
Gut, dry wt per sample:		
0.052 mg	ATP	15.5
0.112 "	"	20.1
0.156 "	"	18.6
Gut with Ca	"	14.5
	"	13.8
Gut without Ca	"	0.5
	"	0.2
Gut	Hexosediphosphate	0.8
	Beta glycerophosphate	0.9
	Ribonucleate	0.1
Gut pH 7.0	ATP	14.7
7.5	"	18.5
8.5	"	24.2
9.0	"	29.3
9.5	"	28.7
10.0	"	29.0

was obtained from Schwartz Laboratories, New York, as the Ca salt which was converted into the sodium salt by addition of the equivalent amount of sodium oxalate. The sodium salt of ribonucleic acid was obtained from the same source. It was prepared from yeast and contained 15.15% N and 9.04% P. Sodium glycerophosphate was prepared from a 25% solution of β-glycerophosphoric acid (Eimer and Amend). The solutions of the substrates were adjusted to a pH of 7-7.5 before use.

Results. The figures for apyrase activity in the organs of *Molgula* are compiled in Table I. The pharynx, the gonads and the excretory organ are low and of the same order of magnitude. The activities in the digestive tract are considerably higher. The highest values were obtained in the intestinal portion; the values for the stomach are significantly lower. The results in Table I show that the amount of inorganic phosphate liberated per mg dry weight is approximately constant for, and thus roughly proportional to, varying tissue concentrations. The activity of the enzyme system is dependent upon the presence of Ca ions and is practically inactive in their absence.

The specificity of the enzyme was tested with β-glycerophosphate, hexosediphosphate and ribonucleate as substrate. When used in a final concentration of 0.02 M and correcting for small blank values obtained with control samples containing substrate without tissue and tissue without substrate, the liberation of inorganic P from these substrates was found to be insignificant compared with ATP. An attempt to characterize the enzyme by the dependence of its activity upon the pH of the solution showed an increase in activity from pH 7 up to a pH of 9, but no significant change was observed on further increase up to a pH of 10. In a preliminary experiment the intestine was homogenized at pH 7.5 in 0.5 M KCl and extracted by centrifuging at 3,000 r.p.m. for 30 minutes. The supernatant was poured into 4 vol. of cold distilled water and a small precipitate was centrifuged off. Both the residue obtained after the first centrifugation as well as the second precipitate were resuspended in small volumes of distilled water and the suspensions tested for enzymatic activity. Only about 20% of the activity of the unfractionated tissue was recovered in the residue while the precipitate was devoid of any activity. This indicates either that the enzyme system is very unstable and inactivated by the extraction, or that the enzyme is more soluble than other animal apyrase preparations such as the myosin fraction of muscle which is one of the richest sources of this enzyme system. The activity of the intestinal homogenate was found to be completely lost after 10 minutes in a boiling water bath.

Discussion. In comparing the apyrase activity in the gut of *Molgula* with that of other tissues, it should be pointed out that the activity in the gut exceeds apparently about twofold the highest activities found so far in animal tissues as well as that of purified myosin.[4,7] This observation and other data

---

[5] Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

of other authors indicate that a high activity of apyrase can be found in tissues which are free of muscular elements and also in cell structures which do not contain myosin (Mitochondria, nucleus).[5,6] However, it should be pointed out that purified myosin splits off only the first one of the phosphate groups of ATP (Adenosinetriphosphatase). Apyrases of other tissues liberate two phosphate groups in the form of inorganic P; but even if half of the inorganic phosphate liberated by *Molgula* intestine were due to the removal of the second phosphate group from ATP the rate at which the first phosphate is split off by homogenates of the gut would still be as high as that of pure myosin. Considering that the dry weight of the intestinal preparations includes the entire cell material and since only a small fraction of it can be responsible for the apyrase activity, its activity in purified state must exceed considerably the activity of purified myosin. The higher activities of apyrases of plant tissues should be mentioned here[8] since a second similarity between plant and ascidian apyrase could possibly be expected in their solubility properties, considering our failure to find intestinal apyrase in the less soluble protein fractions.

The high activity of apyrase in the intestinal cells of *Molgula* suggests an important role of the enzyme in the physiology of this tissue. The mucosa of the intestine of higher animals is known to contain high activities of phosphatases and in this case enzymatic activity was correlated with the phosphorylations of food stuffs in the course of absorption and utilization. However, these phosphatases seem to hydrolyse mono- and di-esters at a much faster rate than ATP[9] while the reverse holds for the enzyme in *Molgula* intestine. Unfortunately, apart from the work of Yonge on *Ciona*[10] very little is known about the physiology and biochemistry of digestion of ascidians and therefore no pertinent suggestion can be made at present as to the possible role of apyrase in the physiology of the intestine of *Molgula*. A possible clue for future investigations of this question can be expected from the difference in the activities of apyrase in the stomach and the mid- and hindgut of this organism.

Summary. The enzymatic liberation of inorganic phosphate from adenosine-triphosphate was tested with homogenates of the pharynx, the gonads, the excretory organ, the stomach and the gut of the tunicate *Molgula manhattensis* (DeKay). The activity in the gut was found to be more than 4 times as high as in the stomach and 20-40 times as high as in the other tissues. The activity in the gut of *Molgula* was compared with the activities observed by other authors in vertebrate tissues and it was found to be twice that observed in mammalian muscle.

---

[6] Steinbach, H. B., and Moog, F., *J. Cell. and Comp. Physiol.*, 1945, **26**, 175.

[7] Bailey, K., *Biochem. J.*, 1942, **36**, 121.

[8] Kalckar, H. M., *J. Biol. Chem.*, 1944, **153**, 355.

[9] Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1943, **149**, 369.

[10] Yonge, C. M., *J. Exp. Biol.*, 1924-25, **2**, 373.

16482 P

Dose Level and Degree of Ketonemia Produced by Growth Hormone and Adrenocorticotropic Hormone.*

LESLIE L. BENNETT, JOSEPH F. GARCIA, AND CHOH HAO LI.

From the Institute of Experimental Biology and the Division of Physiology, University of California, Berkeley.

Previous work[1] from this laboratory has shown that both pure growth hormone and pure adrenocorticotropic hormone will increase the ketonemia and ketonuria of fasted normal rats. In these previous experiments only one dose level of hormone was used. The present experiments have been undertaken to establish whether there was a relationship between the amount of hormone injected and the degree of ketonemia produced.

All animals used were male rats of the Long-Evans strain between 70-76 days of age, weighing between 154-275 g. They were maintained on a stock diet† and were fasted 24 hours prior to the experiment. An initial 0.5 ml of tail blood was obtained under evipal anesthesia. Following this ⅓ of the total dose of hormone was injected intraperitoneally. At 1 and 2 hours later, identical injections of hormone were given and the final 0.5 ml sample of tail blood was taken under nembutal anesthesia 1 hour after the last injection. Blood ketone bodies were determined by the method of Weichselbaum and Somogyi.[2]

The controls consisted of 12 animals which received no injection and of 5 animals which were injected with 3 mg of serum albumin. The hormones used were prepared according to the previously published methods[3,4] and the total doses given were ½ mg, 1½ mg, and 3 mg per rat. There were from 6-9 animals in each experimental group.

Results. The data from the experiments are summarized in Table I. It will be seen that with both hormones there was a significant increase in ketonemia with all dose levels and that there was a graded response with increasing amounts of hormone. When the 2 hormones were given simultaneously at the 1½ mg level the mean effect produced was essentially a summation of the effects of the 2 hormones separately administered. Statistical analysis showed that the increased ketonemia produced by the combination of hormones was significantly greater than that produced by the growth hormone alone, but not greater than that produced by the adrenocorticotropic hormone alone. When the 2 hormones were given simultaneously at the 3 mg level no summation of effects could be demonstrated. This failure to demonstrate summation of effects at the 3 mg level could have been due to the fact that under these conditions the effect of 3 mg of adrenocorticotropic hormone was maximal.

Conclusions. 1. The production of increased ketonemia by either growth hormone or adrenocorticotropic hormone in fasted nor-

---

* Aided by grants from the American Cancer Society (through the National Research Council—Committee on Growth), the National Institute of Health—RG 409, and the Research Board, University of California.

[1] Bennett, L. L., Kreiss, R. E., Li, C. H., and Evans, H. M., *Am. J. Physiol.*, 1948, **152**, 210.

† The diet fed consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, hydrogenated vegetable oil (Crisco or primex) 5.25%. To each kg of diet were added 3.5 g of sardilene (fish oil concentrate containing 3000 USP units of vitamin A and 400 chick units of vitamin D per g).

[2] Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, 1941, **140**, 5.

[3] Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1945, **159**, 355.

[4] Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, **149**, 413.

[5] Fisher, R. A., *Statistical Methods for Research Workers*, 10th Ed., Edinburgh, London, Oliver and Boyd, 1946.

TABLE I.
Effect of GH and ACTH on Blood Ketone Bodies of Fasted Normal Rats.

Injection	Initial blood	3-hr blood	Change	P‖
Controls, no inj. (12)†	7.9 ± 0.94‡	8.0 ± 1.06	+ 0.1 ± 0.85	
3 mg Albumin (5)	6.8 ± 1.45	6.1 ± 1.54	− 0.7 ± 0.18	.55
½ ” ACTH (7)	7.8 ± 1.41	13.0 ± 2.18	+ 5.2 ± 1.83	.01
1½ ” ” (6)	6.9 ± 0.79	16.3 ± 2.52	+ 9.3 ± 2.25	.01
3 ” ” (9)	7.9 ± 1.52	25.6 ± 1.97	+17.7 ± 2.65	.01
½ ” GH (8)	6.4 ± 0.85	10.1 ± 1.25	+ 3.7 ± 0.57	.01
1½ ” ” (7)	7.0 ± 0.84	11.4 ± 1.32	+ 4.4 ± 0.80	.01
3 ” ” (8)	7.4 ± 1.48	16.2 ± 2.00	+ 8.8 ± 1.70	.01
1½ ” ACTH + 1½ mg GH (6)	7.5 ± 0.87	21.5 ± 1.25	+14.0 ± 1.27	.01 .01§ .06*
3 ” ” + 3 ” ” (6)	9.8 ± 1.27	21.5 ± 0.86	+11.8 ± 1.21	.01 .20§ .09*

* Compared with the same dose of ACTH alone.
† Figure in parentheses indicates number of animals.
‡ Standard deviation of the mean.
§ Compared with the same dose of GH alone.
‖ From Fisher's[5] table of t. A value of 0.05 or less is considered significant.

mal rats is confirmed. 2. Under the experimental conditions used, the degree of ketonemia produced is correlated with the amount of hormone administered.

16483

Effect of Age upon Toxicity of Methadon.*

FRANCIS G. HENDERSON AND K. K. CHEN.
From the Lilly Research Laboratories, Indianapolis, Ind.

It has been mentioned that infants are highly susceptible to morphine as compared with adults.[1–3] This statement has been substantiated in animals by Döbeli,[4] Sollmann,[5] Gibbs and Bobb,[6] and Chester and associates.[7] Further, Eddy[8] and Chen and Robbins[9] showed that morphine was more toxic to both very young and aged rabbits, and rats, respectively, as compared with adults. The ages of their animals were accurately recorded.

Methadon is a drug of German origin, picked up by the Technical Industrial Committee[10] of the U. S. Government. Pharmacologic studies by Scott and Chen,[11,12] and

---

* The material used in this investigation was 'Dolophine' (Methadon, Lilly).

[1] Holt, L. E., and Howland, J., *Diseases of Infancy and Childhood*, D. Appleton-Century Company, New York City, 11th ed., 1940, p. 79.

[2] Sollmann, T., *A Manual of Pharmacology*, W. B. Saunders Company, Philadelphia, 6th ed., 1943, p. 285.

[3] Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, The Macmillan Company, New York, 1941, p. 204.

[4] Döbeli, E., *Monatschr. Kinderh.*, 1911, **9**, 397.

[5] Sollmann, T., *Am. J. Physiol.*, 1906, **16**, 1.

[6] Eddy, N. B., *J. Pharm. and Exp. Therap.*, 1939, **66**, 182.

[7] Gibbs, O. S., and Bobb, A., *J. Pharm. and Exp. Therap.*, 1938, **63**, 10.

[8] Chester, A., LaBelle, G. C., and Himwich, H. E., *J. Pharm. and Exp. Therap.*, 1942, **75**, 363.

[9] Chen, K. K., and Robbins, E. B., *J. Am. Pharm. A.*, 1944, **33**, 80.

[10] Kleiderer, E. C., Rice, J. B., Conquest, V., and Williams, J. H., Report No. 981, Office of the Publications Board, Dept. of Commerce, Washington, D. C.

[11] Scott, C. C., and Chen, K. K., *J. Pharm. and Exp. Therap.*, 1946, **87**, 63.

TABLE I.
Median Lethal Dose ± Standard Error of Methadon by Intravenous Injection in Female Albino Rats of Different Ages.

Age		No. of rats injected	LD_{50} ± S.E. mg per kg
Mo.	Days		
	14	40	14.62 ± 1.58
1		39	13.71 ± 1.49
1½		37	10.43 ± 1.19
2		38	11.45 ± 1.26
4		30	10.36 ± 1.30
6		26	13.56 ± 1.83
12		26	10.71 ± 1.45

Isbell and associates[13] reveal that this product has analgesic action and addiction liabilities similar to morphine. For further comparative studies, the effect of age upon the toxicity of methadon was investigated.

Albino rats were used for the toxicity studies with age groups varying from 14 days to 12 months. The animals were of the same sex, females—just as in our morphine work,[9] and from the same source. A total of 26 to 40 rats were used for each age group. The drug was administered by the tail vein. Different dose increments were given for the purpose of determining the median lethal dose (LD_{50}). The LD_{50} was calculated according to the combined slope method of Miller, Bliss, and Braun.[14]

Table I lists the median lethal doses of methadon for the various age groups. There is apparent evidence that rats 14 days and 1 month of age are less susceptible to methadon than those 1½ to 4 months old. Statistical analysis shows this difference to be barely significant at the 5% point. This is just opposite to morphine.[9] Also, rats 12 months old succumbed to methadon in approximately the same dosage as those of 1½ to 4 months of age. Morphine is relatively more toxic to 1-year-old rats than to young adults. For sake of clearness, the LD_{50}'s of methadon for different age groups in the present work are contrasted in Fig. 1 with those of morphine for corresponding age groups published previously.[9]

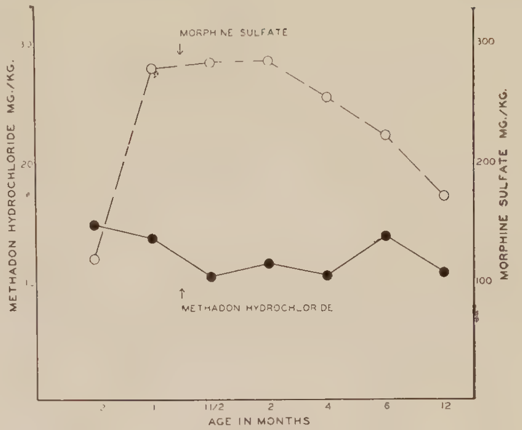


FIG. 1.

Graphic comparison of the effect of age upon the toxicity of morphine and methadon.

Summary. In contrast with morphine, methadon does not show a higher toxicity in very young or 1-year-old rats than in young adults, following intravenous injection.

---

[12] Scott, C. C., Robbins, E. B., and Chen, K. K., *Science*, 1946, **104**, 587.

[13] Isbell, H., Wikler, A., Eddy, N. B., Wilson, J. L., and Moran, C. F., *J. A. M. A.*, 1947, **135**, 888.

[14] Miller, L. C., Bliss, C. I., and Braun, H. A., *J. Am. Pharm. A.*, 1939, **28**, 644.

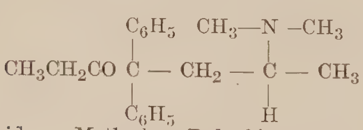
16484

Comparison of Effects of D- and L-Isomers of Amidone and Isoamidone on Cholinesterase.*

MARGARET E. GREIG AND ROBERT S. HOWELL.

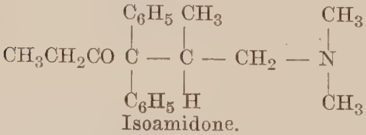
From the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tenn.

It has been observed in experiments *in vivo* that DL-amidone (2-dimethylamino-4, 4-diphenyl-heptanone-5), like morphine, produces certain parasympathomimetic effects such as increased motility of the intestine, decreased heart rate, and salivation.[1,2] Since these actions of morphine may be explained by its ability to inhibit the enzyme cholinesterase[3,4] the effects of the D- and L-isomers of amidone and isoamidone (1-dimethylamino-2-methyl-3, 3-diphenyl hexanone-4) on the cholinesterases of brain and serum have been investigated. The results of these experiments are presented below. The structural formulae of the analgesics being investigated are as follows:



$$CH_3CH_2CO\,\underset{C_6H_5}{\overset{C_6H_5}{C}} - CH_2 - \underset{H}{\overset{CH_3-N-CH_3}{C}} - CH_3$$

Amidone, Methadon, Dolophine or 10820.



$$CH_3CH_2CO\,\underset{C_6H_5}{\overset{C_6H_5}{C}} - \underset{H}{\overset{CH_3}{C}} - CH_2 - \underset{CH_3}{\overset{CH_3}{N}}$$

Isoamidone.

Methods. Cholinesterase activity was measured manometrically. The medium contained $NaHCO_3$ and $MgSO_4$ in concentrations of 0.0249 M and 0.00118 M respectively. The final volume was 2 ml. The gas phase was 95% N_2, 5% CO_2. Acetyl choline was placed in the side bulb of the Warburg vessel and tipped into the main chamber after gassing. The final concentration was 0.01 M. The hydrochlorides of amidone and isoamidone[†] were used and were present in the concentrations indicated in the figures. Rat brain was homogenized in distilled water and diluted to a final concentration of 1 g in 20 ml. One-half ml was used in each vessel. One ml of dog serum was diluted with 2.5 ml water and 0.5 ml of this solution was used in each vessel. The experimental period was 60 minutes and the temperature was 37°C. The values recorded were corrected for the spontaneous hydrolysis of acetyl choline.

Results. Figs. 1 and 2 are a graphic presentation of the effects of the D- and L-isomers of amidone and isoamidone on the cholinesterase of rat brain and dog serum. The serum cholinesterase was found to be inhibited to a greater extent than the brain cholinesterase by both the amidone and isoamidone. D-amidone in a concentration of 0.00087 M inhibited serum cholinesterase by about 60% and brain cholinesterase by about 25%. L-amidone in the same concentrations inhibited these enzymes by approximately 75% and 35% respectively. The effects of isoamidone on these enzymes were similar. Morphine under similar conditions inhibited both the brain and serum cholinesterases to about the same extent, the inhibition being about equal to that of D-amidone on brain cholinesterase. In five experiments with morphine in concentrations of 0.0005 M, 0.00106 M and 0.0021 M, the average inhibitions were 13, 21 and 36% respectively with brain cholinesterase and 17, 27 and 39% with serum cholinesterase.

---

* Funds for carrying out this work were kindly supplied by the Mallinckrodt Chemical Works.

[1] Scott, W. W., Livingstone, H. M., Jacoby, J. J., and Broberg, G. B., *Anesthesia and Analgesia*, 1947, **26**, 18.

[2] Scott, C. C., Chen, K. K., Kohlsteadt, K. G., Robbins, E. B., and Israel, F. W., *J. Pharm. and Exp. Therap.*, 1948, **91**, 147.

[3] Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Therap.*, 1936, **57**, 427.

[4] Eadie, G. S., *J. Biol. Chem.*, 1941, **138**, 597.

[†] Kindly supplied by the Mallinckrodt Chemical Co.

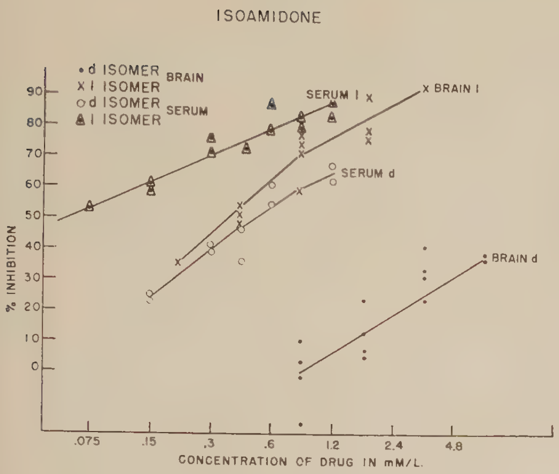


FIG. 1.

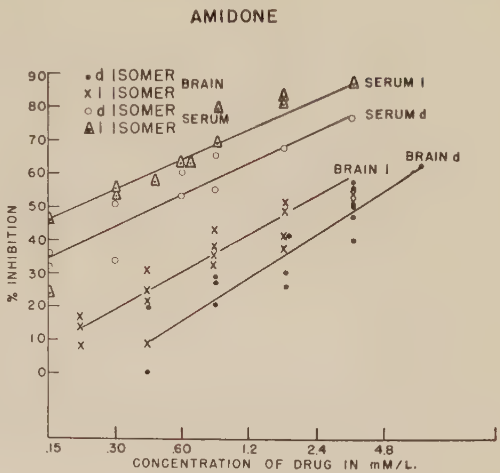


FIG. 2.

In Fig. 1 and 2 the per cent inhibition of cholinesterase, produced by the drug, is plotted against concentration of drug on the logarithmic scale.

The L-isomers of amidone and isoamidone had a greater inhibitory effect than the D-isomers on the cholinesterases of both serum and brain, the differences in effectiveness of the optical isomers being most marked in the case of the action of D- and L-isoamidone on brain cholinesterase. In this case the concentration of the L-isomer required to produce the same inhibition as the corresponding D-isomer was about 1/30 as great. Under our experimental conditions it was not found possible to produce inhibitions of brain cholinesterase of more than 38% with D-isoamidone, probably because of the limitations of solubility of this compound. With serum cholinesterase L-isoamidone was 10-15 times as active as the D-isomer. L-amidone was found to be 1.5 to 2 times as active as the D-isomer with both serum and brain cholinesterase.

Discussion. It has been found that the D- and L-isomers of both amidone and isoamidone inhibit the cholinesterases of rat brain and dog serum. Since this work was begun, an abstract by Eadie, Bernheim and Fitzgerald[5] has appeared in which it was stated that methadon inhibits the cholinesterase of rat brain. Our results confirm this statement. We have found that in the case of brain cholinesterase L-isoamidone is about 30 times as potent as the D-isomer and the L-isomer of amidone is twice as potent as the D-isomer.

In the production of analgesia the L-isomers of both amidone and isoamidone are more effective than the D-isomers but the differences in effect are not as great as those found for cholinesterase, at least in the case of isomers of isoamidone. It has been shown that the analgesic morphine,[6] demerol,[2] and amidone[1,2] exhibit parasympathomimetic effects *in vivo,* and morphine, codeine, dilaudid, desomorphine,[7] amidone, isoamidone, antipyrine, and certain derivatives of antipyrine[8] inhibit cholinesterase *in vitro.* Whether there is a correlation between cholinesterase inhibition and analgesic activity is not known. Certain drugs seem to possess definite parasympathomimetic action without exhibiting analgesic activity, but one such, namely prostigmine, has been shown by Slaughter and Munsell[9] to potentiate the effect of morphine in raising the threshold of pressure pain responses in cats. It might be of interest in this connection to determine whether physostigmine, the central actions of which are opposed to those of prostigmine, also potentiates the analgesic action of morphine.

---

[5] Eadie, G. S., Bernheim, F., and Fitzgerald, D. B., *Fed. Proc.,* 1948, **7**, 216.

[6] Bernheim, F., *The Interaction of Drugs and Cell Catalysts,* Burgess Publishing Company, 1942, p. 11.

[7] Wright, C. I., and Sabine, J. C., *J. Pharm. and Exp. Therap.,* 1943, **78**, 375.

[8] Zeller, E. A., *C. R. Soc. Physiol. Suisses,* 1942, **20**, 51.

[9] Slaughter, D., and Munsell, D. W., *J. Pharm. and Exp. Therap.,* 1940, **68**, 104.

Summary. 1. The D- and L-isomers of amidone and isoamidone inhibit the cholinesterases of rat brain and dog serum. 2. These drugs exert a greater inhibitory action on serum cholinesterase than on brain cholinesterase. 3. The L-isomers are more effective than the D-isomers in inhibiting these enzymes. 4. The greatest difference in effect between the D- and L-isomers was observed in experiments on the effect of D- and L-isoamidone on brain cholinesterase.

16485

Histochemical Demonstration of Sites of Choline Esterase Activity.*

GEORGE GOMORI.

From the Department of Medicine, The University of Chicago.

Glick[1] has shown that choline esters of higher fatty acids are hydrolyzed by choline esterase at an appreciable rate. Since fatty acids from C_{10} up form highly insoluble Ca, Ba and Co salts, the histochemical demonstration of choline esterase activity appeared feasible, provided the enzyme could survive the rigors of fixation and embedding.

Experimental. Previously, lauroyl-, myristoyl-, palmitoyl-, and stearoyl choline chloride had been synthesized by the addition of trimethylamine onto the corresponding β-chloroethyl esters.[2] For the present experiments the compounds were prepared by direct esterification of choline chloride with the corresponding acyl chlorides. An equimolar mixture of choline chloride and acyl chloride was heated to about 130 to 140°C under constant stirring. Evolution of HCl ceased after about 1 hour, and the mixture solidified into a thick paste. This mass was washed with petroleum ether and subsequently extracted by refluxing it with acetone. On cooling (sometimes in the ice box) the ester chloride precipitated in the form of a white crystalline solid. It was purified by 2 more recrystallizations from boiling acetone. It was impossible to determine the exact melting points because all esters became semifluid just above 58°C. Their purity was checked by analysis for N, Cl, and, after hydrolysis by 0.5 N alkali at 56°C, for fatty acid.

The rates of hydrolysis of the various esters by choline esterase were determined by the following methods:

Acetylcholine. A 0.01 M solution of acetylcholine bromide in 0.1 M phosphate buffer of pH 8 was prepared. 25 ml samples of the buffered substrate were placed in 3 flasks. To one of the flasks 4 drops of a 0.01 M solution of prostigmine bromide was added; this flask served as a control. One-half to 1 ml of tissue homogenate (usual ratio, 1:10 to 1:20) or serum was added to all flasks which were subsequently incubated for 1 hour at 37°C. At the end of this period, hydrolysis was stopped by the addition of 4 drops of prostigmine solution. The flasks were cooled to room temperature and titrated with 0.025 N NaOH to the same blue shade (pH ± 9.2), thymol blue being used as an indicator.

Lauroyl-, etc. choline. 0.002 M solutions of the substrates in 0.05 M tris(hydroxymethyl)-aminomethane-HCl buffer[3] of pH 8 were prepared. Ten ml samples of the buffered substrates were pipetted into 3 centrifuge tubes.

---

* This work has beeen done under a grant from the Douglas Smith Foundation for Medical Research of The University of Chicago.

[1] Glick, D., *J. Biol. Chem.*, 1941, **137**, 357.

[2] Fourneau, E., and Page, H. J., *Bull. soc. chim. de France*, 1914, **15**, 544.

[3] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 33.

TABLE I.
Data of Analysis of Choline Ester Chlorides.

Ester	N%		Cl%		Fatty acid mEq/g	
	Theor.	Found	Theor.	Found	Theor.	Found
Lauroyl	4.35	4.16	11.03	10.33	3.11	3.35
Myristoyl	4.00	3.85	10.15	9.99	2.86	2.85
Palmitoyl	3.71	3.63	9.4	9.3	2.65	2.78
Stearoyl	3.46	3.53	8.75	8.64	2.47	2.35

TABLE II.
Rates of Enzymatic Hydrolysis of Choline Esters.
(Micromoles of ester hydrolyzed in 1 hr by 1 g of tissue or 1 ml of serum or enzyme preparation.)

Enzyme	Acetyl	Lauroyl	Myristoyl	Palmitoyl	Stearoyl
Dog serum	100	6.7	8.3	0.5	0
Dog adrenal	225	186	62	12	not done
Mouse brain	675	36	11.4	2.5	12.5
Rat brain	375	7.7	5	3.6	0
Pigeon brain	450	40	31.6	28	21.5
Purified bovine choline esterase (Winthrop); 1 mg	475	1.6	0	0	0

To one of the tubes 2 drops of prostigmine solution were added. One-half to 1 ml of tissue homogenate or serum (dialyzed against normal saline for 4 hours to remove the bulk of Ca) was added to each of the tubes which were subsequently incubated for 1 hour at 37°C. At the end of this period, hydrolysis was stopped by the addition of 2 drops of prostigmine solution. The tubes were spun for 10 minutes at 2400 rpm. Five ml samples were transferred to colorimetric tubes, and 5 ml of a 0.2% solution of $CaCl_2$ in 1.5% gelatin was added to each of them. The turbidity resulting from Ca soaps was determined with a photoelectric colorimeter, the sample incubated in the presence of prostigmine being used as a blank. The amount of fatty acid liberated was read from a curve previously calibrated with solutions of Na laureate, containing 1 to 12 μM of salt in 5 ml.

It is clearly realized that this latter method is not suitable for exact studies since part of the fatty acid liberated is precipitated by Ca and Mg in the tissues and another part may be adsorbed to the tissue fragments. However, it served as a useful guide for the approximate estimation of rates of hydrolysis, and its results were in good agreement with histological findings.

As shown, most of the crude enzymes used hydrolyze higher choline esters at an appreciable rate, although the pattern of relative rates varies considerably according to the source of the enzyme. In fact, a highly purified enzyme preparation of the electric organ of *Torpedo* (generously contributed by Dr. D. Nachmansohn of the Department of Neurology, Columbia University), although extremely active towards acetylcholine (4700 μM/ml/hr), failed to hydrolyze any of the higher esters.

By the use of Coujard's technic,[4] it could be proven that choline esterase survives, at least to some extent, the procedures of fixation and embedding. With a clean steel pen marks were made on microscopic slides, dog serum and homogenates tested in previous experiments being used as ink. The slides were dried rapidly and immersed in chilled acetone for 12 hours, subsequently treated with cold alcohol-ether mixture for 2 hours and finally with xylene for 1 hour at 56°C. The slides were then coated with a thin film of collodion and incubated for 2 to 12 hours at 37°C in a 0.002 M solution of myristoylcholine, buffered to pH 8 with tris(hydroxymethyl)-aminomethane and containing 0.01 M $CaCl_2$. When the slides were removed from the mixture and examined under the microscope, a thick deposit of typical crystals of calcium myristate was seen at the sites of the marks. No deposition of crystals could be observed at the marks made with brain homogenates if eserine or prostigmine was added to the incubating mixture in a concentration of 10^{-5} M, while in the marks made with serum, enzymatic activity was greatly reduced but not completely abolished.

---

[4] Coujard, R., *Bull. d'hist. appl.*, 1943, **20**, 161.

On the basis of these findings a histochemical technic for the visualization of sites of choline esterase activity could be worked out. At first it was attempted to duplicate the technic for lipase[5] in all details except for the different substrate but after many trials it became apparent that conditions must be modified in several respects. The following technic, which is not to be considered final, gave good results in the overwhelming majority of cases. Rapid fixation seems to be critical since often only the more superficial layers of the block were entirely satisfactory while in the depth the preservation of the enzyme was spotty.

1. Fix and embed tissues the same way as in the lipase technic, except for a minor modification in embedding which improves the cutting qualities of the block. After acetone dehydration, run tissues through a/, a mixture of equal parts of absolute alcohol and ether (1 to 3 hours); b/, 4% collodion in alcohol-ether (12 hours); c/, 2 changes of chloroform, 1 hour each. All steps up to c/ should be performed at ice box temperature. From chloroform, embed in paraffin. Bring sections, as in the technic for lipase, to distilled water.

2. Incubate slides in the following mixture for from 2 to 16 hours at 37°C:

Stock solution I. 0.1 M maleate-tris(hydroxymethyl)-aminomethane buffer pH 7.5-7.7, 50 to 100 ml; 0.1 M cobaltous acetate solution, 30 to 50 ml; distilled water to make 300 ml; add about 1 mg each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

TABLE III.
Maleate-tris(hydroxymethyl)-aminomethane buffer.

ml 0.5N NaOH	pH	ml 0.5N NaOH	pH
1	5.08	9	6.86
2	5.30	10	7.20
3	5.52	11	7.50
4	5.70	12	7.75
5	5.88	13	7.97
6	6.05	14	8.15
7	6.27	15	8.30
8	6.50	16	8.45

Preparation of the buffer. Dissolve 29 g of maleic acid and 30.4 g of tris(hydroxymethyl)-aminomethane in 500 ml of water, decolorize with a few g of charcoal and filter. Ten ml of this stock solution + the indicated number of ml of 0.5 N NaOH, diluted to a total of 50 ml, will give 0.1 M buffers of pH values from 5.08 to 8.45 at 23°C.

Stock solution II. A 0.02 M solution of choline ester in distilled water. For most purposes the myristoyl ester is the most satisfactory; it requires an incubation time of about 6 hours. Both stock solutions, with a crystal of camphor added, will keep in the ice box indefinitely. For use, add 1 ml of stock solution II to 50 ml of stock solution I, previously heated to 37 to 40°C. At this temperature the mixtures should be perfectly clear, except in the case of the stearoyl ester which is not readily soluble in the presence of salts.

3. Wash slides under the tap for about 2 minutes.

4. Immerse them in a dilute solution of light yellow ammonium sulfide (a few drops to 50 ml of distilled water) for about 15 minutes. Darkening of sites of enzyme activity is very gradual, especially if palmitoyl choline was used as a substrate.

5. Wash slides under the tap. Counterstain as desired; dehydrate, clear and mount. Sites of choline esterase activity are black.

At first, in analogy to the technic for lipase, Ca, later Sr, Ba and Mg were tried instead of Co. However, all of them were found to give very coarse crystalline precipitates while Co gives a fine granular precipitate. The localization of the enzyme and the intensity of the reaction were the same, regardless of the metal ion used. When used in the technic for lipase, Co causes a moderate but noticeable inhibition of the enzyme in certain locations. $CaCl_2$, $MgCl_2$ and $MnCl_2$ were added to the incubating mixture because their metal ions activate choline esterase *in vitro*[6] and seem to intensify the picture also in the histochemical experiment. The buffer used was chosen because, in comparison with other buffers (tris-(hydroxymethyl)-aminomethane-HCl, collidine), it seemed to inhibit the enzyme very little and to prevent the nonspecific impregnation of the tissues by Co which at the relatively high pH level of optimal enzymatic activity may be quite troublesome. The low substrate concentration was adopted because it was found that higher concentrations of substrate (over 0.001 M) inhibit the enzyme. Similar inhibition in even lower ranges of substrate concentration was observed by Mendel and Rudney,[7] and by Alles and Hawes.[8]

---

[5] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 362.

So far only a limited number of tissues were examined by the new technic. The presence of choline esterase could be ascertained in many tissues. Only some of the most interesting locations will be mentioned here: sympathetic ganglia in all species examined; brain of the pigeon, the dog (especially in the basal ganglia), the mouse (especially certain nuclei of the oblongata; Fig. 1); smooth muscle of the gastrointestinal tract, urinary bladder and uterus in several species; the conducting system in the dog's heart (Fig. 2); structures in the striated muscle of the mouse and the pigeon, tentatively identified as muscle spindles and not as motor end plates since they lie in the septa, separated by connective tissue from the muscle fibers proper (Fig. 3); adrenal medulla in some species. The reaction is completely inhibited in most tissues by the addition of prostigmine in a concentration of 10^{-5} M to the incubating mixture.

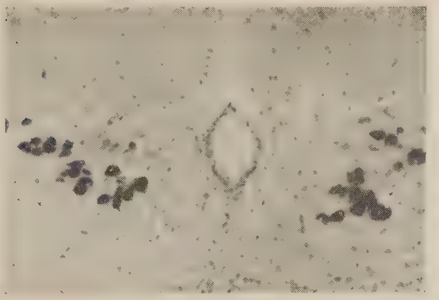


FIG. 1.
Brain of the mouse. A group of ganglion cells (nucleus of XIIth nerve?) stained selectively. Substrate: myristoyl-choline.

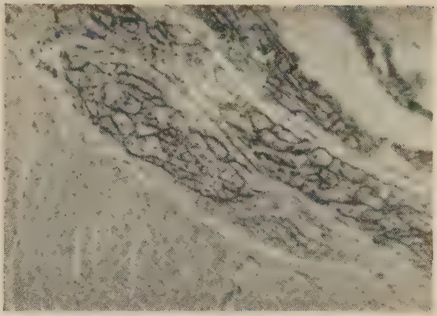


FIG. 2.
Conducting system of the dog's heart. Substrate: myristoyl-choline.

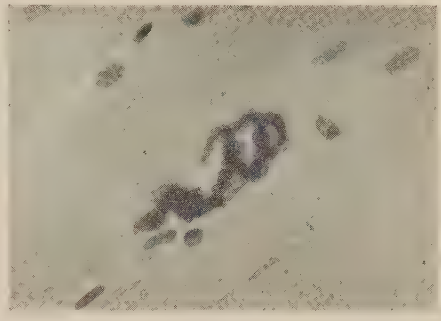


FIG. 3.
Typical structure in muscle of the mouse. Substrate: myristoyl-choline.

Besides the demonstration of enzymatic activity at the specific locations mentioned, the following observations of interest were made:

1. There is a considerable difference in the pattern of distribution of the enzyme in various species although the sympathetic nervous system or parts of it are stained in most species.

2. The finer distribution of the enzyme differs according to the substrate used. With lauroylcholine the enzyme presents a granular appearance and is often found in nuclei while

---

[6] Nachmansohn, D., *Nature*, 1940, **145**, 513.

[7] Mendel, B., and Rudney, H., *Bioch. J.*, 1943, **37**, 59.

[8] Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, 1940, **133**, 375.

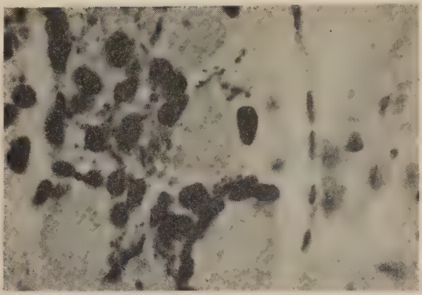


FIG. 4.
Sympathetic ganglion of the rabbit. Substrate: lauroyl-choline. Nuclei of satellite cells and granular material stained.

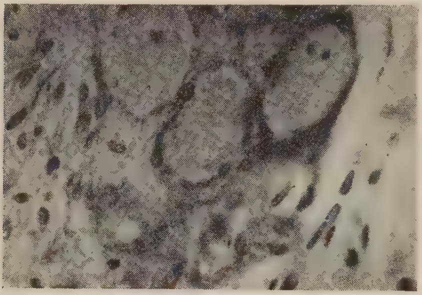


FIG. 5.
Another section of the same tissue block. Substrate: palmitoyl-choline. Nerve fibers around ganglion cells stained.

with palmitoylcholine it appears to be distributed in a uniform way mainly in nerve fibers. The most striking example is the sympathetic ganglia in which lauroylcholine produces an intense staining of the nuclei of the satellite cells (Fig. 4) while palmitoylcholine outlines the nerve fibers, the satellite cells being left entirely unstained (Fig. 5). This difference may indicate the existence of 2 (or more) different enzymes.

3. There is a species preference for certain substrates. Dog and mouse tissues give much more intense pictures with lauroyl- and myristoylcholine than with palmitoylcholine while for human and pigeon tissues the reverse holds true. Rat and guinea pig tissues fail to hydrolyze any of the substrates at a rate sufficiently high to give clearcut results.

Summary. Some choline esterases hydrolyze higher choline esters readily and they also survive to a demonstrable extent the procedures of histological fixation and embedding. It is possible to demonstrate them histochemically by incubating slides with the substrate in the presence of a cobalt salt; at the sites of choline esterase activity insoluble cobalt-soap will precipitate which can be transformed into black CoS. The histochemical distribution and properties of choline esterase are described.

16486

Characteristics of the Protein Groups Associated with the Hypotensive Activity of Trypsin.*

DONALD E. BOWMAN.†

From the Department of Biochemistry and Pharmacology, Indiana University School of Medicine, Indianapolis.

In view of the action of trypsin and parenteral trypsin-like proteases and their inhibitors in certain types of shock,[1-5] blood coagulation[6-12] and other fields it is desirable to char-

---

[*] This work was supported by a grant from the Office of Naval Research of the United States Navy. Read in part before the American Society of Biological Chemists in Atlantic City, March 19, 1948.

[†] The author is grateful to Miss Marianne Daries and Mr. Richard Moore for technical assistance.

[1] Rocha e Silva, M., *Arquivos d. Inst. Biol.*, 1939, **10**, 93.

[2] Dragstedt, C. A., and Wells, J. A., *Quart. Bull. Northwestern Univ.*, 1944, **18**, 104.

[3] Burdon, K. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 24.

[4] Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.

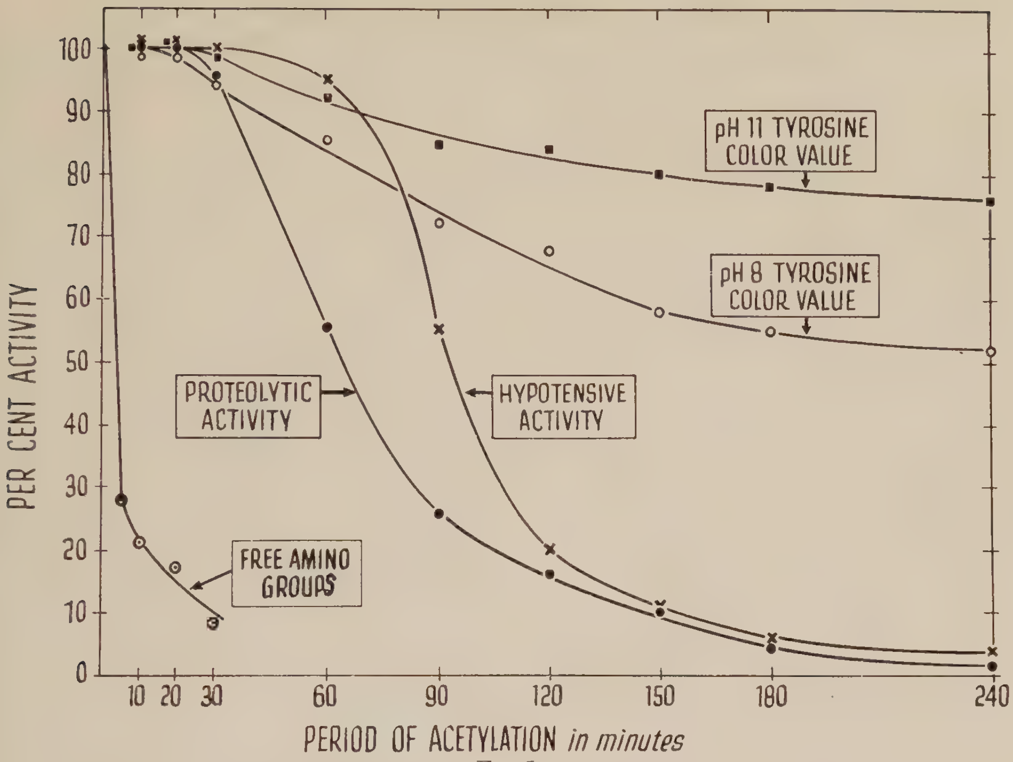


Fig. 1.

Tyrosine color value, free amino groups, and proteolytic and hypotensive activities of acetylated trypsin.

acterize the protein groups associated with the hypotensive effect of intravenously administered trypsin and to determine whether the hypotensive activity is due to its proteolytic activity. Findings relative to these points are given in the present report.

Methods. Hypotensive activity was determined in the usual manner of recording the carotid blood pressure in dogs. The per cent of normal activity was calculated as 100 times the reciprocal of the weight of the treated enzyme in mg per kg body weight required to cause a fall in blood pressure approximating the duration and degree of fall resulting from the injection of 1 mg of untreated trypsin per kg before and after the injection of the treated material. Injections were made into the femoral vein.

Proteolytic activity was estimated by determining the relative amount of a given preparation required to accomplish the same degree of digestion as a standard amount of untreated trypsin required for 50% hydrolysis of casein at pH 7 and 38°C in 1 hour.

Acetylation was carried out with ketene produced by pyrolysis of acetone. The pH during acetylation was maintained between 6 and 7 by the addition of sodium hydroxide. Iodination was carried out in 0.8 M phosphate

---

[5] Scroggie, A. E., Jaques, L. B., and Rocha e Silva, M., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 326.

[6] Douglas, S. R., and Colebrook, L., *Lancet*, 1916, **2**, 180.

[7] Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, 1936, **20**, 543.

[8] Tyson, T. L., and West, R., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 494.

[9] Ferguson, J. H., and Erickson, B. N., *Am. J. Physiol.*, 1939, **126**, 661.

[10] Tagnon, H. J., and Soulier, J. P., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 440.

[11] Croxatto, H., *Rev. soc. argentina biol.*, 1946, **22**, 477.

[12] Macfarlane, R. G., *J. Physiol.*, 1947, **106**, 104.

buffer at pH 7.4 avoiding a large excess of free iodine at any time. Amino nitrogen was estimated by the method of Van Slyke[13] and the tyrosine-tryptophane color values were determined by the pH 8 and the pH 11 methods of Herriott.[14] Difco Laboratories crude trypsin, 1:250, was used.

Results. That free amino groups are not essential to the proteolytic or hypotensive activity of trypsin may be observed following the acetylation of the enzyme. After 30 minutes treatment with ketene the amino nitrogen was observed to decrease to 8% of its original value yet the proteolytic and hypotensive activities were essentially unchanged. This is shown in Fig. 1. As further indicated in this figure, following more prolonged acetylation essentially complete loss of hypotensive and proteolytic properties is observed with a decrease in tyrosine-tryptophane color value to about one-half of its original level.

It is seen that following treatment of the acetylated enzyme with alkali at pH 11, the tyrosine-tryptophane color values are only partially restored. With more prolonged treatment for as long as 4 hours at pH 9, 10, or 11, no further restoration of color value was observed. In repeated observations, in comparison with non-acetylated controls subjected to the same variations in pH, no significant restoration of hypotensive activity was observed as the result of alkaline hydrolysis of the acetylated enzyme.

It was originally thought that partial recovery of proteolytic activity follows hydrolysis at pH 11. However, since this is not observed at pH 9.5 or 10 it is believed that in the original observations there may have been greater loss of activity in the controls due to pH alone than there was in the acetylated preparations. Therefore, in view of the lack of recovery following this mild alkaline treatment it appears that the phenolic hydroxyl groups are not essential to either the hypotensive or the proteolytic activity of trypsin. That the amino and phenolic groups of trypsin are not essential for proteolytic activity is in agreement with the recent findings of Fraenkel-Conrat and associates.[15]

After treating trypsin with 1% formaldehyde at pH 11 for 1 hour the average values of dialysed and lyophilized preparations for proteolytic and hypotensive activities were respectively 52% and 14% of the corresponding values of the control preparations which were maintained at the same pH without formaldehyde.

Greater loss of hypotensive than proteolytic activity as the result of iodination has been described.[16] However, it may be further noted that after reacting with 2.0×10^{-3} m Eq of iodine per mg, about half of the saturating amount of iodine, which is sufficient to reduce the hypotensive effect to 2 to 5% of its original level, the tyrosine-tryptophane color value of trypsin may be 95% of that of the untreated material.

From the foregoing it would appear that trypsin shock as well as proteolytic activity is associated with groups which acetylate with ketene but the resulting acetyl linkages do not hydrolyze in dilute alkaline solution. There is a rapid loss of hypotensive effect upon treating with formaldehyde at pH 11. Iodination, which greatly decreases the hypotensive effect, is associated with only a slight decline in tyrosine-tryptophane color value.

Indole, guanidyl, and amide groups react rapidly with formaldehyde under the conditions employed.[18,19] However, of these 3 the indole groups iodinate with retention of considerable reducing power. Anson[20] has pointed out that tryptophane treated in 0.01 N acetic acid with 3 Eq of iodine per mole has the same color value as untreated tryptophane. Under

---

[13] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, Baltimore, 1932.

[14] Herriott, R. M., *J. Gen. Physiol.*, 1935, **19**, 283.

[15] Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H., *Fed. Proc.*, 1948, **7**, 155.

[16] Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 408.

[17] Herriott, R. M., *J. Gen. Physiol.*, 1941, **25**, 185.

[18] Fraenkel-Conrat, H., Brandon, B. A., and Olcott, H. S., *J. Biol. Chem.*, 1947, **168**, 99.

[19] Olcott, H. S., and Fraenkel-Conrat, H., *Chem. Rev.*, 1947, **41**, 151.

[20] Anson, M. L., *J. Gen. Physiol.*, 1940, **23**, 321.

TABLE I.
Influence of Bean Trypsin Inhibitors on the Hypotensive Activity of Trypsin.

		Amount		Hypotensive effect	
Dog No.	Materials injected	Inhibitor mg/kg	Trypsin mg/kg	Fall in blood pressure mm	Duration min.
1	Navy bean inhibitor alone	12,200	0	0	0
1	Soy bean inhibitor fraction* alone	42	0	12	1
2	Trypsin alone	0	2.5	44	9
2	Navy bean inhibitor incubated with trypsin at 25° 11 hrs	2.5	2.5	38	10
3	Trypsin alone	1	0	34	2.2
3	Soy bean inhibitor fraction* incubated with trypsin at 25° 10 min	1	1	36	2.0
4	Trypsin alone	0	15	60	45
4	Navy bean inhibitor inj. immediately before trypsin	12,000	15	57	30

* First alcohol-insoluble soy bean trypsin inhibitor (crude).[25]

the conditions of iodination followed in the present work, treatment with 2 Eq and 4 Eq of iodine per mole yielded color values equivalent to 77 and 83% of the original value respectively. It is of interest to note that reaction with formaldehyde which, like iodination, decreases proteolytic activity less than the hypotensive effect reduces the color value only 10 to 20%.

Herriott[14] has found that treatment of tryptophane or glycyl tryptophane with ketene results in a considerable loss in tyrosine-tryptophane color value with the ratio of the pH 8 to pH 11 values ranging from 0.6 to 0.73 for glycyl tryptophane and from 1.0 to 1.2 for free tryptophane. It is noted that this ratio for acetylated trypsin is within the limits given for glycyl tryptophane. A marked decrease in the tyrosine-tryptophane color value upon acetylating free tryptophane and lack of hydrolysis in alkaline solution was also observed under the conditions of acetylation and alkaline treatment used in the present work.

Therefore, while all of the characteristics of the groups associated with the hypotensive effect of trypsin are not limited to a single protein group, the single free amino acid which appears to best satisfy the requirements so far defined is tryptophane. However, it cannot be said with certainty that the properties of the indole structure are the same in a protein molecule as they are in the free amino acid. In a footnote Brand and Kassell[21] have given the tryptophane content of trypsin as 4.6%. Our values for the commercial trypsin used have been somewhat less.

In earlier reports it was pointed out that the hypotensive activity of iodinated trypsin does not parallel its proteolytic[16] or thromboplastic[22] activity. That proteolytic and hypotensive activities are not always decreased equally is further indicated by work with the trypsin inhibitors of beans. The preparation and differentiation of the inhibitors used and their inhibition of proteolysis have been reported.[16,23–25] However, as shown in Table I, the same inhibitors failed to have significant influence on the hypotensive effect of trypsin in dogs. It is observed that injection of inhibitors, with or without previous incubation with the enzyme prior to injection, does not significantly alter the hypotensive effect of the trypsin. Furthermore, the established slow rate of recovery of blood pressure in dogs following the injection of 5 to 15 mg of trypsin per kg was not altered by the injection of 10 times as much inhibitor during the recovery period. However, it should be added that the navy bean trypsin inhibitor does protect rabbits against the acute lethal effect of trypsin. The latter is to be described elsewhere.

---

[21] Brand, E., and Kassell, B., *J. Biol. Chem.*, 1942, **145**, 359.

[22] Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 262.

[23] Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 139.

[24] Bowman, D. E., *Fed. Proc.*, 1945, **4**, 84.

[25] Bowman, D. E., *Arch. Biochem.*, 1948, **16**, 109.

Summary. Free amino and phenolic groups do not appear to be essential to the proteolytic or the hypotensive activity of trypsin. Characteristics of the protein groups associated with the hypotensive activity of trypsin are given. These correspond with the properties of free tryptophane although participation of other groups is not excluded. Changes in the proteolytic and hypotensive activities of trypsin are not always parallel.

16487 P

On the Relative Efficacy of Emetine in Intestinal and Hepatic Amebiasis.

LEO G. PARMER.*

From the Pharmacology Section, Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

Although emetine has been used therapeutically in amebiasis for more than 35 years, the tissue distribution and concentration of this alkaloid have not been reported. Nevertheless, certain limitations of the drug's efficacy are well known. Emetine is considered curative in amebic hepatitis and even in amebic abscess. On the other hand, relapses of amebic dysentery or the recurrence of cysts in the stool are frequently seen after emetine therapy despite the ability of the drug to clear up the acute symptoms.[1]

There are various theories as to why emetine is ineffective in amebic dysentery and curative in amebic liver disease. One widely accepted theory is based on observations that, *in vitro*, emetine is capable of destroying the trophozoites but not the cysts of *Endameba histolytica*. Cysts are as a rule not found in tissues; their origin in the intestinal lumen is in trophozoites leaving the tissues and encysting. According to this theory, emetine is ineffective because both trophozoites and cysts occur in amebic dysentery. On the other hand, since only trophozoites are present in amebic liver disease this type of amebiasis is readily eradicated by emetine. Craig believes that emetine fails to eradicate all the trophozoites from the intestine, leaving some to start the disease process again.[1] No mention is made as to why the drug is capable of destroying the trophozoites in the liver.

TABLE I.
Liver and Intestinal Concentration of Emetine After an Intramuscular Injection of 6 mg per kg. Concentrations are averages of 3 rabbits in each group.

Rabbit group	Time after inj.	Emetine conc. (mg/kg)	
		Liver	Intestine
1	1 hr	24.9	5.5
2	12 ”	41.6	1.7
3	3 days	19.2	1.7
4	4 ”	15.4	1.2
5	6 ”	10.3	0
5	13 ”	2.7	0
7	20 ”	2.1	0
8	28 ”	1.0	0

The role of blood and tissue levels of chemotherapeutic agents as a factor in effectiveness of therapy has assumed great importance in recent years. The following findings are presented to show that the tissue levels of emetine may explain the divergent results obtained with the drug in amebiasis of the liver and of the intestine.

Method. Each of 24 albino rabbits weighing about 2.5 kg were injected intramuscularly with 6 mg per kg of emetine hydrochloride in aqueous solution. They were then sacrificed in groups of 3 at intervals of from 1 hour to

---

* Present address: Medical Division, Food and Drug Administration, Washington, D.C.

[1] Craig, C. F., *Etiology, Diagnosis, and Treatment of Amebiasis*, Williams & Wilkins Co., Baltimore, 1944.

28 days. A portion of the large intestinal wall, freed of feces, and a portion of liver were analyzed for emetine. The analysis was adapted from the general method of Brodie *et al.*[2]

Table I shows the emetine concentration in the liver and intestine. One hour after injection, the concentration of drug was much higher in the liver than in the intestine. At 12 hours, the liver concentration of drug was 41.6 mg per kg compared to the intestinal concentration of only 1.7 mg per kg. At the end of 4 days the low concentration in the intestine became almost undetectable whereas that of the liver was maintained for more than 4 weeks.

Summary. Injection of emetine into rabbits was followed by a relatively high drug concentration in the liver for more than a month, whereas the level in the intestine was always much lower and became undetectable after 4 days. The findings of a high concentration and prolonged presence of emetine in the liver and a low concentration and transient presence of the drug in the intestine are proposed as the explanation for the efficacy of emetine in amebic liver disease and the drug's failure in amebic intestinal disease in man.

---

[2] Brodie, B. B., Undenfriend, S., and Dill, W., *J. Biol. Chem.*, 1947, **168**, 335.

16488

Phospholipid Utilization in the Diabetic Dog.*

Paul V. Harper, Jr., William B. Neal, Jr., and Georgiana R. Rogers.
(Introduced by Lester R. Dragstedt.)
From the Department of Surgery, University of Chicago.

Depancreatized dogs maintained on insulin developed fatty livers, emaciation, and anemia.[1] Dragstedt maintains that this syndrome is due to the lack of a specific substance present in pancreas, which he has named lipocaic.[2] The syndrome is relieved or prevented by feeding choline, raw pancreas, and various choline-free pancreatic extracts.

It has been shown that the plasma phospholipid level is lowered in lipocaic deficiency.[3] Phospholipid is probably the chemically active form of fat and the significant lipid fraction in the transport of fat. There is evidence that choline deficiency in rats, which resembles lipocaic deficiency, results in an impairment of transportation of fat from the liver to the tissues.[4] In the present study, the plasma phospholipid turnover was measured in normal dogs and in diabetic dogs under a variety of conditions.

Numerous studies on phospholipid metabolism have been performed with the use of isotopic tracers. One conclusion from this work is that the plasma phospholipid is produced largely in the liver.[5]

In the present study, it is assumed that the plasma phospholipid is produced in the liver and withdrawn from the plasma for utilization by the tissues. Since the phospholipid levels remained constant during the period of measurement, the rate of production of new phos-

---

* This work has been aided by grants from the Rockefeller Foundation—Postwar Training of Medical Veterans Fund, Mr. Andrew E. Wigeland, and the Kenneth G. Smith and Douglas Smith Foundations for Medical Research of The University of Chicago.

[1] Allan, F. N., Bowie, J. J., MacLeod, J., Jr., and Robinson, W. L., *Brit. J. Exp. Path.*, 1924, **5**, 75.

[2] Dragstedt, L. R., Prohaska, J. V., and Harms, H. P., *Am. J. Physiol.*, 1936, **117**, 175.

[3] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

[4] Stetten, D., Jr., and Salcedo, J., *J. Biol. Chem.*, 1944, **156**, 27.

[5] Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, 1943, **150**, 47.

TABLE I.
Fasting Normal Dogs.

Dog No.	Total plasma lipid (mg%)	Plasma lipid phosphorus (mg%)	Half-life of injected phospholipid (hr)	Utilization (mg% lipid phosphorus disappearing per hr)	Fraction of plasma phospholipid utilized per hr	Remarks
234	479	11.8	5.6	1.46	.124	
769	900	17.8	5.5	2.20	.126	
S-892	1020	21.1	6.8	2.16	.101	
965	450	8.4	5.0	1.17	.139	
913	—	17.5	7.2	1.67	.096	
913	—	12.2	7.0	1.21	.099	
913	—	17.1	5.7	2.08	.122	Fed
944	—	11.0	7.6	1.22	.091	
966	427	7.8	5.4	1.54	.128	Fed
1B	—	12.2	6.5	1.31	.107	Zilversmit's dogs[7]
2B	—	11.6	5.0	1.61	.139	
3B	—	17.9	5.4	2.29	.128	
Mean		13.8	6.0	1.66	.114	
Standard error of mean		±1.2	±.26	±.12	±.005	

pholipid equaled the rate of disappearance or utilization.

Methods. The rate of disappearance was measured by the method of Zilversmit *et al.*[6,7] with little modification. Donor dogs were injected subcutaneously with 2 to 3 millicuries of radioactive phosphorus as sodium phosphate, and 24 hours later the dogs were heparinized and bled and the plasma was separated. From 64 to 81% of the plasma radioactive phosphorus was in the phospholipid fraction and the remainder was inorganic phosphorus. The plasma containing the tagged phospholipid was injected intravenously into 7 normal and 12 diabetic dogs in the postabsorptive state. After allowing 20 to 30 minutes for complete mixing, blood samples were drawn at intervals of 1 to 2 hours, and the radioactivity and phosphorus content were determined on the trichloracetic acid precipitate of the plasma. All samples had activities of 3 to 20 times background activity and were counted for appropriate periods. Phosphorus was determined by the method of Fiske and Subbarow.[8] Plasma lipids were determined by the method previously reported from this laboratory.[9] All dogs were fasted before the determination for 18 to 20 hours. Half the daily insulin requirement plus 25 g of glucose in skimmed milk was given to all but the normal and control groups half an hour before the injection of the phospholipid. Two hundred g of raw pancreas a day for periods up to several weeks were administered to one group. All diabetic dogs were controlled with insulin on a regime previously reported from this laboratory.[9]

The fact that all the injected radioactive phosphorus was not in the form of phospholipid did not alter the findings measurably. The formation of new plasma phospholipid by the liver from injected inorganic radioactive phosphorus does not, in our experience, reach levels comparable to those observed in the present study (5 to 13 counts per second per 3 cc sample) until as much as 20 microcuries/kg are given, and even then not until 12 to 24 hours have elapsed. Since all the present experiments were terminated within 6 hours, and since the inorganic radioactive phosphorus injected in the plasma amounted to only .02 to .04 microcuries/kg it is evident that this factor may be neglected. The inorganic radioactive phosphorus in the plasma

---

[6] Zilversmit, D. B., Entenman, C., and Fishler, M. C., *J. Gen. Physiol.*, 1943, **26**, 325.

[7] Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, 1943, **26**, 333.

[8] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

[9] Dragstedt, L. R., Donovan, P. B., Clark, D. E., Goodpasture, W. C., and Vermeulen, C. W., *Am. J. Physiol.*, 1939, **127**, 755.

did not interfere with the determination of the radioactivity of the plasma phospholipid phosphorus as it was washed out with trichloracetic acid.

In all cases the disappearance of the injected phospholipid from the blood stream was found to follow a logarithmic curve. The time required for half the tagged phospholipid to disappear, or the half-life, was measured graphically from the plot of the logarithm of the radioactivity of the trichloracetic acid precipitate of the plasma against time. From this the utilization was calculated from the relation:

$$\text{Utilization} = \frac{\text{plasma lipid phosphorus (mg\%)}}{\text{half-life (hr)} \times 1.44} =$$

mg% lipid phosphorus disappearing per hour from the blood stream.

The fraction of the total plasma phospholipid disappearing per hour equals utilization / plasma lipid phosphorus (mg%) or

$$\frac{1}{\text{half-life (hr)} \times 1.44}$$

The standard error of the mean for the various groups was calculated from the formula for small samples:

$$S_x = \frac{\sigma}{\sqrt{N-1}}$$

Results. The results for normal dogs are summarized in Table I. The striking finding in this group was the relative constancy of the fraction of plasma phospholipid utilized per hour in spite of wide variations in the plasma phospholipid concentration among the various dogs. This suggests that utilization of phospholipids is roughly proportional to its concentration. Tables II and III show the results for diabetic dogs fasting and given insulin. It is seen that no essential difference exists between the two groups. Comparing these two groups to the normal group, the usual changes of lipocaic deficiency are present in varying degrees. On the average, there is a decreased plasma lipid and plasma lipid phosphorus. The injected phospholipid disappears at a significantly greater rate in the diabetic groups, the half-life averaging 4.7 and 4.8 hours against 6.0 hours for the normal dogs. However, because of the lower phospholipid level in the plasma of the diabetic dogs, the

TABLE II.
Fasting Diabetic Dogs.

Dog No.	Type of preparation	Daily insulin requirement (units)	Total plasma lipid (mg%)	Plasma lipid phosphorus (mg%)	Half-life of injected phospholipid (hr)	Utilization (mg lipid phosphorus disappearing per hr)	Fraction of plasma phospholipid utilized per hr	Remarks
930	Total pancreatectomy	10	500	8.8	3.7	1.66	.188	10 wks postop. No suppl. 5 wks
934	”	34	570	11.5	4.8	1.66	.145	3 wks postop.
801	Partial pancreatectomy with ligation of duct	32	450	11.2	5.0	1.56	.139	136 wks postop. No supp. 9 wks
892	Same	4	290	7.9	5.4	1.02	.129	38 wks postop. No suppl. 20 wks
922	Pancreatic duct ligation	4	213	8.1	5.0	1.12	.139	19 wks postop.
	Mean	17	406	9.7	4.8	1.40	.148	
	Standard error of mean		±66	±.2	±.3	±.14	±.010	

TABLE III.
Diabetic Dogs Given Insulin and Sugar.

Dog No.	Type of preparation	Daily insulin requirement (units)	Total plasma lipid (mg%)	Plasma lipid phosphorus (mg%)	Half life of injected phospholipid (hr)	Utilization (mg% lipid phosphorus disappearing per hr)	Fraction of plasma phospholipid utilized per hr	Remarks
919	Total pancreatectomy	64	770	9.1	3.6	1.74	.192	30 wks postop. No suppl. 1 wk
933	,, ,,	38	467	9.0	4.9	1.27	.141	10 wks postop. No suppl.
933	,, ,,	28	325	5.4	6.0	.63	.116	14 wks postop. No suppl.
934	,, ,,	24	490	11.5	4.7	1.70	.147	8 wks postop. No suppl.
890	,, ,,	68	650	11.4	5.9	1.33	.118	40 wks postop. No suppl. 18 wks
489	Partial pancreatectomy with ligation of duct	44	830	15.3	5.2	2.04	.133	102 wks postop. No suppl. 15 wks
892	Same	4	240	4.7	3.5	.93	.198	44 wks postop. No suppl. 26 wks
895	Same	4	310	12.4	4.0	2.16	.173	40 wks postop. No suppl. 5 wks
923	Pancreatic duct ligation	4	455	11.4	4.4	1.81	.158	25 wks postop. No suppl.
	Mean	35	505	10.0	4.7	1.50	.153	
	Standard error of mean		±66	±1.1	±.3	±.16	±.011	

absolute utilization of plasma phospholipid is slightly less in the diabetic groups. The fraction of plasma phospholipid utilized per hour, however, is increased in the diabetic dog, indicating that for a given plasma level, phospholipid is utilized more rapidly in the diabetic dog, regardless of whether he is getting insulin; and conversely, for a given rate of utilization, the diabetic dog has a lower plasma lipid phosphorus. Again, as in the normal dog, there is a relatively wide fluctuation in the absolute utilization which parallels the phosphorus level, while the fraction of plasma phospholipid utilized per hour is relatively constant.

Upon administration of raw pancreas to the diabetic dogs, the plasma lipid and lipid phosphorus level rose to normal, as shown in Table IV, but the utilization of phospholipid rose well above the normal level, and the fraction utilized per hour was higher in this group. The half-life of the injected phospholipid was shorter.

Table V shows the results in one dog with a subtotal pancreatectomy with the remnant of pancreas remaining attached to a duct. In this preparation, the very high utilization of phospholipid parallels the high lipid phosphorus level. However, the fraction utilized per hour is in the same range as the other diabetic dogs. The effect of insulin and sugar on the fraction of the phospholipid utilized per hour is negligible.

Preliminary results on 3 diabetic dogs given 2 g of choline chloride a day for 3 days showed effects similar to the group fed raw pancreas. Plasma lipids averaged 451 mg%; plasma lipid phosphorus averaged 9.2 mg%; half-life of injected phospholipid averaged 4.0 hours; utilization averaged 1.70 mg% per hour; and the fraction of phospholipid utilized per hour averaged .177, the highest of all groups. No significant effect was observed in two fasting normal dogs given similar amounts of choline.

It is apparent from the data that in both the normal and the diabetic dog the rate of utilization of phospholipid is proportional to the concentration of phospholipid in the plasma. Two dogs on which determinations were made under a variety of conditions illus-

TABLE IV.

Diabetic Dogs Given 200 Grams per Day Raw Pancreas, Insulin, and Sugar.

Dog No.	Type of preparation	Daily insulin requirement (units)	Total plasma lipid (mg%)	Plasma lipid phosphorus (mg%)	Half-life of injected phospholipid (hr)	Utilization (mg% lipid phosphorus disappearing per hr)	Fraction of plasma phospholipid utilized per hr	Time on raw pancreas (wks)	Remarks
933	Total Pancreatectomy	62	960	11.3	3.6	2.17	.192	1	15 wks postop.
933	” ”	92	1300	16.6	3.3	3.50	.210	5	19 ” ”
919	” ”	58	930	16.7	5.0	2.32	.139	2	35 ” ”
919	” ”	96	1100	12.8	4.4	2.02	.158	3	28 ” ”
934	” ”	42	1300	18.7	4.6	2.82	.151	5	15 ” ”
801	Partial pancreatectomy with ligation of duct	40	1470	18.4	5.7	2.24	.122	5	145 ” ”
892	Same	12	480	8.8	3.7	1.65	.187	2	46 ” ”
892	Same	10	747	13.8	3.8	2.52	.182	5	50 ” ”
	Mean	65	1030	14.7	4.2	2.42	.168		
	Standard error of mean		±84	±.4	±.3	±.22	±.011		

TABLE V.
Subtotal Pancreatectomy with Remnant on Duct—No Supplement.

Daily insulin requirement (units)	Total plasma lipid (mg%)	Plasma lipid phosphorus (mg%)	Half-life of injected phospholipid (hr)	Utilization (mg% lipid phosphorus disappearing per hr)	Fraction of plasma phospholipid utilized per hr	Remarks
50	1450	23.7	5.0	3.30	.139	Insulin and sugar. 3 wks postop.
94	1850	30.1	4.8	4.35	.145	Fasted 6 wks postop.

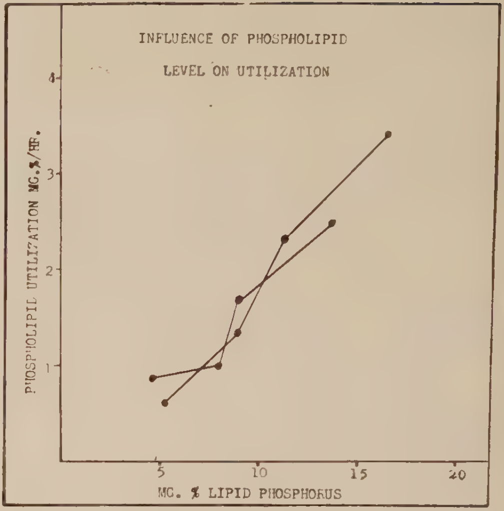


FIG. 1.

trate this point particularly well (Fig. 1). This confirms the conclusion arrived at by Balfour[10] from less direct evidence, and is entirely consistent with the observations of Flock and Bollman[11] on chickens. This concept permits a broader interpretation of the significance of the plasma lipid phosphorus level.

It is also apparent that the utilization of plasma phospholipid is altered after production of diabetes by pancreatectomy in such a manner that phospholipid disappears from the plasma more rapidly in the diabetic dog than in the normal; that is to say, the fraction of the circulating plasma phospholipid utilized per hour is significantly increased in the diabetic animals. This effect seems to be unchanged whether the animal is fasted or given insulin and sugar and may be slightly increased when the animal is fed raw pancreas. The significance of this alteration in the phospholipid metabolism is not clear, especially as it seems to be aggravated instead of corrected by feeding raw pancreas and by choline.

Summary and conclusions. Phospholipid tagged with radioactive phosphorus was injected intravenously into normal and diabetic dogs. From the rate of disappearance of the tagged phospholipid from the blood stream and the plasma lipid phosphorus level, the rate of utilization of phospholipid was calculated. It was observed in both normal and diabetic groups that the rate of utilization was roughly proportional to the plasma lipid phosphorus concentration. The absolute utilization of plasma phospholipid was slightly lower in the diabetic group, but the proportion of plasma phospholipid utilized per hour was significantly higher in this group. Administration of insulin and sugar to diabetic dogs caused no significant change. Administration of raw pancreas to diabetic dogs increased the absolute utilization very markedly and the proportion utilized per hour only slightly.

---

[10] Balfour, W. M., *Gastroenterology,* 1947, **9**, 686.

[11] Flock, E. V., and Bollman, J. L., *J. Biol. Chem.,* 1944, **156**, 151.

16489

Effect of Celiac Ganglionectomy upon Experimental Peptic Ulcer Formation.*

C. W. LILLEHEI[†] AND OWEN H. WANGENSTEEN.

From the Department of Surgery, University of Minnesota Hospitals, Minneapolis, Minn.

In a recent report,[1] we have investigated the effect on the gastrointestinal tract of complete excision of the celiac, superior, and inferior mesenteric ganglia. Extirpation of these ganglia removes essentially the postganglionic sympathetic fibers to the gastrointestinal tract from the stomach through the colon and results in a severe bloody diarrhea associated with typical appearing peptic ulcers in somewhat less than 50% of the dogs. These ulcers are located for the most part in the stomach or duodenum, but occasionally as far down as the terminal ileum or colon. Likewise, these ulcers often progress to hemorrhage or perforation. The question has been raised by ourselves as well as others as to whether these ulcers are an effect of the removal of the postganglionic sympathetic innervation or whether they develop as the consequence of a state of protein deficiency and emaciation which several investigators[2,3] have shown to be often associated with peptic ulcer in dogs.

This investigation was undertaken to appraise what specific effects, if any, complete excision of the postganglionic sympathetic innervation to the stomach and duodenum has upon susceptibility to gastroduodenal ulceration.

Experiment. Male and female dogs of medium size, previously dewormed and between the ages of 1½ to 5 years were used in these experiments. The animals were divided into 3 series as follows: Series I, dogs in which complete excision of the celiac ganglia was performed leaving undisturbed the vagi nerves and the superior and inferior mesenteric ganglia. This operation removes the postganglionic sympathetic innervation of the stomach, duodenum, and also that of the liver, spleen, and pancreas as well. Series II, consists of dogs in which in addition to complete excision of the celiac ganglia, the superior mesenteric ganglia were removed also. This operation removes the postganglionic sympathetic fibers to the stomach, duodenum, and small intestine. Series III consists of a control series of normal dogs of comparable age and size.

Operative procedure. For ganglionectomy intravenous nembutal anesthesia was used, sterile technic, and a midline abdominal in-

---

* Supported by a grant from the Surgical Study Section of the United States Public Health Service, the John and Mary R. Markle Foundation, and the Graduate School of the University of Minnesota.

[†] National Cancer Trainee.

[1] Lillehei, C. W., Dixon, J. L., Friesen, S. R., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**.

[2] Weech, A. A., and Paige, B. H., *Am. J. Path.*, 1937, **13**, 249.

[3] Keller, A. D., *Arch. Path.*, 1936, **21**, 127.

TABLE I.
Ulcer Production Following Celiac Ganglionectomy.
(30 mg histamine-base in beeswax intramuscularly daily.)

Dog No.	Wt, lb	Days postop. before hist. injections	No. of hist. inj.	Results
1	22	3	3	Died, perforated duodenal ulcers
2	32	3	2	Died, perforated duodenal ulcers, gastric ulcers
3	32	6	5	Died, perforated duodenal ulcer
4	27	41	5	Sacrificed, large gastric ulcer
5	27	45	5	Sacrificed, antral and duodenal ulcer
6	33	58	4	Sacrificed, moribund, 3 duodenal ulcers
7	38	64	3	Died, gastric ulcer, perforated duodenal ulcers
8	28½	108	5	Sacrificed, perforated duodenal ulcer, antral hemorrhages
9	46	165	5	Sacrificed, duodenal ulcers, antral hemorrhages
10	31	167	5	Sacrificed, multiple penetrating duodenal ulcers, antral hemorrhages
11	29½	181	5	Died, perforated duodenal ulcers
12	36	499	5	Sacrificed, penetrating duodenal ulcer, antral erosions

No. of dogs injected	12
No. of dogs with ulcer	12
No. of dogs with perforated ulcer	6

TABLE II.
Ulcer Production Following Celiac and Superior Mesenteric Ganglionectomy.
(30 mg histamine-base in beeswax intramuscularly daily.)

Dog No.	Wt, lb	Days postop. before hist. injections	No. of hist. inj.	Results
13	25	21	2	Died, multiple gastric erosions, severe esophageal ulceration
14	36	127	1	Died, perforated esophageal ulcers, erosion of mediastinal structures by gastric juice
15	34	210	4	Died, large (3 x 2 cm) perforated duodenal ulcer

No. of dogs injected	3
No. of dogs with ulcer	3
No. of dogs with perforated ulcer	2

cision. Upon entering the abdomen the stomach was retracted to the far right, exposing the celiac axis. The left semilunar ganglion may then be seen through the peritoneum and palpated as a firm flat button about ½ cm in diameter lying between the celiac artery and the left adrenal. The right semilunar ganglion lies deeper between the celiac artery and the vena cava. The removal of just the right and left semilunar ganglia fails to insure a complete deprivation of the sympathetic innervation to the stomach and duodenum. This observation is based on the fact that microscopic examination of serial sections along the celiac axis in such animals after a simple removal of the semilunar ganglia shows an abundancy of ganglion cells remaining in smaller or larger groups along the vessel wall. However, if the heavy bundles of nerve fibers which form a sheath around the celiac, hepatic, left gastric, and splenic arteries are removed in addition to the semilunar ganglia a much more complete sympathectomy is achieved. Therefore, in performing celiac ganglionectomy on these dogs we have followed this latter procedure of dissecting free and removing the ganglia and the heavy bundles of ensheathing nerve fibers from the celiac, splenic, hepatic, and left gastric arteries from the aorta to a point 7 to 8 cm distally. The ganglia and these nerve fibers are then removed in one piece leaving the vessel walls entirely clean. The same procedure was applied to the superior mesenteric artery in those dogs in which the superior mesenteric ganglia were removed.

TABLE III.
Production of Ulcer in Normal Dogs.
(30 mg histamine-base in beeswax daily.)
Control Series.

No. of dogs	No. histamine injections	No. dogs with Ulcer	No. dogs with Erosion
21	5	2*	1

* No perforations.

Method of study. In the operated animals, a period following surgery varying from 3 days to 16½ months as indicated in Tables I and II was allowed to elapse before the daily injections of histamine-in-beeswax were begun as a stimulus to ulcer formation. The technic of preparing the histamine-in-beeswax mixture has been described in detail elsewhere.[4] Histamine injections were not instituted on any animals that appeared sick. All animals in Series I, II, and III were given not more than 5 consecutive daily intramuscular injections of 30 mg of histamine-base-in-wax. Those animals not succumbing to ulcer formation beforehand were sacrificed for autopsy by the administration of nembutal 18 hours after their fifth histamine injection. Obviously moribund animals were sacrificed earlier in order to obtain fresh autopsy specimens.

All dogs were fed the same stock diet consisting of 300 g of dry dog biscuit,[‡] 250 cc of pasteurized milk, and 200 g of fresh horse meat daily at 9:30 A.M. The daily histamine injections were given at 4:00 P.M. and at that time the feed pans were removed from the cages and the dog's food intake for the day recorded. The histamine-in-beeswax is prepared fresh once each week, and each batch is tested before use for potency by its effect on the gastric secretion of a pouch dog. By observance of these conditions and the use of dogs of the same relative age,[5] ulcer production by this method will be found to be consistent, so that in a group of healthy normal dogs of medium size and age, typical peptic ulcers will develop in an average period of 23 days[4] and likewise most normal animals will not show gastroduodenal ulceration after just 5 consecutive histamine injections.

Results. A. Celiac Ganglionectomy. There is a certain immediate mortality related to the operative procedure essentially from hemorrhage. However, dogs surviving the operation recover quickly and without special care. These dogs are able to eat the regular stock diet beginning the first postoperative day and do not manifest any gastrointestinal disturbances such as vomiting or diarrhea. The stools are normal in character and contain no gross blood.

Table I indicates the incidence of gastroduodenal ulceration occurring in this group of dogs with their celiac ganglia removed when histamine-in-wax injections are instituted. Table I is to be compared with the incidence of ulceration in normal dogs (Table III) given a like amount of histamine. The symptoms of a developing ulcer were usually easily recognized and did not differ in the two groups. Such animals, when developing an ulcer, became listless, refused to eat, and often bloody stools or vomitus were observed.

B. Celiac and Superior Mesenteric Ganglionectomy. Following the removal of these ganglia, the dogs invariably exhibit a severe bloody diarrhea only slightly less severe than previously reported[1] for animals with celiac, superior and inferior mesenteric ganglionectomy.

Six animals had celiacectomy with superior mesenteric ganglionectomy. Three of these animals died in the postoperative period as a result of the severe diarrhea, inanition, and pathological changes in the gastrointestinal tract before histamine-in-beeswax injections

---

[4] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gyn. and Obst.*, 1942, **75**, 170.

[‡] Standard Brands, Inc., N.Y.—Vitamin D_2 fortified.

[5] Lillehei, C. W., and Wangensteen, O. H., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 129.

TABLE IV.
Summary of Pathologic Findings in Dogs Dying After Celiac and Superior Mesenteric Ganglionectomy. (No histamine injections.)

Dog No.	Postoperative Day of death	Wt (lb) Initial	Wt (lb) Final	Autopsy findings
16	56	27	11¾	Entire G. I. T. filled with blood from multiple bleeding gastric ulcers and erosions
17	16	31	19	Multiple bleeding erosions in stomach, duodenum, sm. intestine, severe hyperemia of intervening mucosa
18	11	34	24	Hemorrhagic gastritis, duodenitis, and enteritis, pneumonia

were begun. The survival period and autopsy findings for these animals are summarized in Table IV. Likewise, the severe weight losses experienced by these animals are to be noted. The other three animals, subjected to celiac and superior mesenteric ganglionectomy, following recovery from the effects of the operation were given histamine-in-beeswax injections as indicated in Table II. The extreme ulcer susceptibility of this group is evident.

Discussion. The essential physiologic changes occurring within the gastrointestinal tract after postganglionic sympathectomy have been commented on by several investigators. These changes are hypersecretion (Thomsen),[6] hyperemia (Laignel-Lavastine),[7] and hypermotility (Koennecke and Meyer;[8] Lium).[9] The occurrence of any of these changes would be expected to predispose to ulcer formation. Furthermore, our own studies tend to confirm the physiologic changes noted by other observers after postganglionic sympathectomy of the gastrointestinal tract. The above results would likewise suggest that the postganglionic sympathetic fibers of the gastrointestinal have a mitigating effect upon vagus action under normal conditions.

Moreover, it would not seem premature on the basis of our own studies to suggest that there appears to be a real difference in the physiologic effects of pre- and postganglionic sympathectomy on the gastrointestinal tract.

Conclusion. 1. Postganglionic sympathectomy of the gastrointestinal tract potentiates the susceptibility to the histamine-induced ulcer in dogs. 2. This increased susceptibility to ulcer formation has continued over the longest interval during which a dog was observed, in one instance for 499 days following celiac ganglionectomy.

---

[6] Thomsen, E., *Acta Medica Scand.*, 1924-25, **61**, 377.

[7] Laignel-Lavastine, M., *Recherces Sur Plexus Solaire,* Paris, 1903, Georges Steinheil.

[8] Koennecke, W., and Meyer, H., *Mitteilungen, Aus den Grenzebieten der Medizin und Chirurgie,* 1922, **35**, 297.

[9] Lium, R., *Surgery,* 1941, **9**, 538.

16490

Potentiating Effect of *Nocardia asteroides* on Sensitization to Picryl Chloride and on Production of Isoallergic Encephalomyelitis.

Jules Freund and Murray M. Lipton.

From the Division of Applied Immunology, the Public Health Research Institute of the City of New York, Inc.

It has been shown that antibody formation is enhanced when antigens are administered in water-in-oil emulsions and that the effect on antibody formation is not associated with a conspicuous alteration of sensitization. The addition of killed mycobacteria to antigens in water-in-oil emulsions, however, has two effects; it alters sensitization and further potentiates antibody formation.[1] Both of these effects as caused by living *M. tuberculosis* in tuberculous animals had been observed by Lewis and Loomis,[2] and Dienes.[3]

Examples of the potentiating effect of killed mycobacteria in water-in-oil emulsions are numerous. They include with regard to sensitization the delayed type (tuberculin type) of sensitization to equine serum,[4] contact type of sensitization to picryl chloride[5] and the production of isoallergic encephalomyelitis;[6] with regard to antibody formation the production of agglutinins against *S. typhosa,*[7] neutralizing antibodies against influenza virus[8] and neutralizing and complement fixing antibodies against rabies virus.[9] The relationship of adjuvants to immunization to malaria is particularly interesting. It is possible to immunize rhesus monkeys against infection with *P. knowlesi* by killed parasites in water-in-oil emulsion containing killed *M. tuberculosis* but identical vaccine without mycobacteria is ineffective. Furthermore, a lipid extract from tubercle bacilli which enhances antibody formation against *S. typhosa* is ineffective as a substitute for the whole mycobacterial cell with regard to immunization against malaria.[10] This observation increased our interest in the nature of the synergistic effect of mycobacteria.

It is hoped that light may be shed on the mode of action of mycobacteria and on immune-response in general by finding other agents endowed with similar adjuvant properties. The purpose of the present paper is to report on the effect of killed *Nocardia asteroides* (actinomyces) on sensitization to picryl chloride and the production of isoallergic encephalitis.

Sensitization to picryl chloride. Landsteiner and Chase[5] found that the skin of the guinea pig can be sensitized (contact type of sensitization) to this simple compound by injecting a picryl conjugate of guinea pig stromata into the peritoneal cavity, provided that killed tubercle bacilli in paraffin oil were likewise injected into the peritoneal cavity; later, the sensitization was found to be more satisfactorily attained by a single subcu-

---

[1] Freund, J., and Walter, A. W., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 47; Freund, J., *Ann. Rev. Microbiology,* 1947, **1**, 291.

[2] Lewis, P. A., and Loomis, D., *J. Exp. Med.,* 1924, **40**, 503.

[3] Dienes, L., *J. Immunol.,* 1928, **15**, 153.

[4] Freund, J., and McDermott, K., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 548.

[5] Landsteiner, K., and Chase, M. W., *J. Exp. Med.,* 1940, **71**, 237.

[6] Morgan, I. M., *J. Exp. Med.,* 1947, **85**, 131; Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.,* 1947, **85**, 117; Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.,* 1947, **57**, 179; Kopeloff, L. M., and Kopeloff, N., *J. Immunol.,* 1947, **57**, 229; Morrison, L. A., *Arch. Neur. and Psych.,* 1947, **58**, 391.

[7] Freund, J., and Bonanto, M. V., *J. Immunol.,* 1944, **48**, 325.

[8] Friedewald, W. F., *J. Exp. Med.,* 1944, **80**, 477.

[9] Freund, J., and Lipton, M. M., unpublished experiments.

[10] Freund, J., Thomson, K. J., Sommer, H. E., Walter, A. W., and Pisani, T. M., *Am. J. Trop. Med.,* 1948, **28**, 1; Freund, J., Thomson, K. J., Hough, H. B., and Pisani, T. M., *J. Immunol.,* in press.

TABLE I.
Sensitization to Picryl Chloride.

Material injected	Group A	Group B	Group C
Picryl stromata	10 mg	10 mg	10 mg
Saline solution	0.6 ml	0.6 ml	0.6 ml
Falba	0.5 "	0.5 "	0.5 "
Paraffin oil	0.6 "	0.6 "	0.6 "
N. asteroides	5 mg	1 mg	0 mg
Guinea pig No.			
1	5+	5+	±
2	5+	5+	tr.
3	5+	5+	0
4	4+	5+	0
5	4+	±	0
6	2+	trace	0
7	±		0
8	±		0
9			0
10			0

5+ = bright red, markedly elevated.
4+ = pink or bright pink, elevated.
2+ = pale pink, slightly elevated.
± = faint pink, small spots.
trace = very faint pink, small spots.
0 = no discoloration, no elevation.

taneous injection of picryl guinea pig stromata incorporated into a water-in-oil emulsion containing killed tubercle bacilli.[11] No sensitization (or occasionally a feeble one) occurs in the absence of mycobacteria.

To test the effect of *N. asteroides,* guinea pigs were injected with picryl guinea pig stromata* emulsified in paraffin oil containing killed fungi instead of mycobacteria. The strain of *N. asteroides* (American Type Culture Collection No. 9969) is not acid-fast by the Ziehl-Neelsen technic but retains carbol fuchsin when treated with 1% aqueous H_2SO_4 and washed with water. The fungi were collected from Sabouraud's agar, autoclaved and dried over P_2O_5. The animals received 5 simultaneous deep subcutaneous injections, a total of 1.7 ml of the emulsions; the material injected is given in Table I. The technic of the preparation of emulsion was given in previous papers.[6] Sensitization was tested 31 days after the injection by placing 1 drop of a 1% picryl chloride solution in olive oil on the skin. The reactions were read 1 and 2 days later. Table I shows that killed *N. asteroides* was effective as a potentiating agent.

Production of isoallergic meningo-encephalitis. Rivers and associates[12] succeeded in producing allergic encephalitis by frequently repeated injections of brain extracts or suspensions of autolysed brain without adjuvants. A single injection of normal brain is, however, sufficient if the brain is incorporated in a water-in-oil emulsion containing killed *M. tuberculosis* or *M. butyricum.*[6] In the absence of mycobacteria the rapid appearance of the phenomenon fails to occur and certain lipid extracts from tubercle bacilli are ineffective in this respect.[13]

To test the effect of *N. asteroides* as a substitute for mycobacteria, 3 groups of guinea pigs were given 3 simultaneous deep subcutaneous injections of fresh guinea pig brain in water-in-oil emulsion containing killed fungi.

The material injected and the results are given in Table II. *N. asteroides* was at least as effective as *M. tuberculosis* or *M. butyricum.* The neurological symptoms and histological lesions of the central nervous system were similar to those found in guinea pigs injected with brain, paraffin oil and killed mycobacteria.[6] Groups of 18 mice received 0.2 ml of an emulsion containing 0.08 ml of a 10% suspension of mouse brain in saline, 0.04 ml Falba, 0.08 ml paraffin oil and 0.2 mg (dry weight) killed *N. asteroides,* or the same material but rabbit brain instead of mouse brain. No neurological symptoms of encephalitis were observed during 1 month as in similar experiments with mycobacteria.[6]

Sensitization to N. asteroides and to tuberculin. It can be expected that the killed fungi suspended in paraffin oil will produce specific fungi was extracted with 30 ml of 1/20 skin sensitization of the delayed type. The sensitization was tested with an alkaline extract of the partially defatted fungi. Fluid medium cultures were treated with 0.1%

---

[11] Chase, M. W., personal communication.

* Obtained through the courtesy of Dr. Merrill W. Chase.

[12] Rivers, T. M., Sprunt, D. M., and Berry, G. P., *J. Exp. Med.,* 1933, **58**, 39; Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.,* 1934, **60**, 559; Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.,* 1935, **61**, 689.

[13] Freund, J., and Lipton, M. M., unpublished experiments.

TABLE II.
Isoallergic Meningo-encephalitis in Guinea Pigs.

Material injected per guinea pig		Guinea pig No.[*]	Paralysis day of onset	Paresis day of onset	Dead or killed *in extremis*, days	Histological lesions in CNS
Guinea pig brain	0.1 g	1	23		K25	Present
Saline sol.	0.9 ml	2	22		K25	’’
Falba	0.5 ’’	3	24		K25	’’
Paraffin oil	1 ’’	4	24		K25	’’
N. asteroides	10 mg	5		24[†]	Living[‡]	
		6		24[†]	’’ [‡]	
		7	36		K43	Absent
		8		56 and 122[†]	Living[‡]	
		9		25[†]	’’ [‡]	
		10			’’ [‡]	
		11			D62	’’
		12	20		K24	Present
		13			D38	Absent
		14	13		K19	Present
Guinea pig brain	0.1 g	15			D22	Absent
Saline sol.	0.9 ml	16		24	D27	Present
Falba	0.5 ml	17	19		K20	’’
Paraffin oil	1 ’’	18		16	K16	’’
N. asteroides	1.0 mg	19	23		K24	’’
		20	19		K24	’’
		21		15	D16	’’
		22	20		K22	’’
		23	28		K31	’’

[*] Group I, No. 1 through 10; Group II, No. 11 through 14; Group III, No. 15 through 23.
[†] Transient.
[‡] 133 days.

formalin, the fungi extracted with acetone, later with equal parts of 95% ethanol and ether and dried over H_2SO_4. One gm of these fungi was extracted with 30 ml of 1/20 normal NaOH for 2 hours and centrifugalized. The supernate was neutralized with 1 normal HCl.

The guinea pigs in the picryl chloride experiment were skin-tested 6 weeks after the original injection. All except the controls (Group C) reacted to a 1:2 dilution of the alkaline extract with redness, elevation and necrosis. The reactions varied from about 20 to 30 mm in diameter and lasted for more than 2 days. It may be noted that the aqueous extract produced a slight redness in the skin of the controls but this reaction diminished during the first day. Eleven weeks after the original injection 5 animals of the encephalitis experiment were skin-tested with 0.1 ml saline containing 0.1 mg killed *N. asteroides*. The reactions were severe and were of the delayed type.

Besides the successful use of *N. asteroides* reported in the present paper allergic encephalitis has been produced with the aid of mycobacteria, *M. tuberculosis* and *M. butyricum*. Both are equally effective and both sensitize to tuberculin although the latter to a lesser degree than the former.

It was desirable to learn whether guinea pigs injected with *N. asteroides*, an actinomycete biologically close to mycobacteria, would likewise be made sensitive to tuberculin. Drake and Henrici[14] reported that living *Nocardia* suspended in paraffin oil do not induce sensitization to tuberculin, although previously Goyal[15] had found that strains of *Nocardia* sensitize guinea pigs to tuberculin. Goyal does not mention the degree of sensitization. For this investigation, skin-tests were made with an Old Tuberculin prepared from a culture grown on a synthetic medium[†] and with an Old Tuberculin prepared from a glycerin veal infusion broth; the test doses ranged from 0.1 ml of a 1:20 dilution to 0.1 ml of a 1:100 dilution. Actually, it was found that killed *Nocardia* do sensitize to tuberculin, but the sensitization is usually feeble; it is necessary to use rather high concentrations of tuberculin and to make the test some time (5 or 6 weeks) after the primary sensitizing injection.

---

[14] Drake, C. H., and Henrici, A. T., *Am. Rev. Tub.*, 1943, **48**, 184.

[15] Goyal, R. K., *Ann. Inst. Pasteur*, 1937, **59**, 94.

[†] This tuberculin, prepared from growths of human strains of tubercle bacilli on Dorset's Synthetic Medium, was sent to Dr. M. W. Chase from the Reichel Division of Wyeth, Inc., through the courtesy of Dr. B. Scott Fritz.

Details of the reactions follow: All of the picryl chloride animals which received fungi reacted to a 1:20 dilution of old tuberculin, prepared on a synthetic medium. Seven weeks after the injection of the emulsion the following reactions were observed: After 24 hours in Groups A and B pinkishness and elevation ranging from 10 mm to 20 mm in diameter with blanching; at 48 hours the reactions ranged from negative to ill-defined pinkishness and elevation 18 mm in diameter; controls (Group C) had very slight reactions at 24 hours which became negative at 48 hours. The same OT was diluted 1:80 and 4 guinea pigs of groups A and B were tested. The reactions at 24 hours ranged from very ill-defined pinkishness 10 mm to well-defined redness and induration 14 mm in diameter. At 48 hours the reactions were doubtful to ill-defined faint pinkishness and elevation 12 mm in diameter. Eight guinea pigs of groups A and B were tested with a 1:20 dilution of OT prepared on broth. The reactions were stronger and some of the animals had necrotic reactions.

The animals of the encephalitis experiment were tuberculin-tested at different intervals of time following the injection of brain and fungi and oil. After 21 days Groups II and III and after 44 days 5 survivors in Group I failed to react to a 1:20 dilution of OT. The latter 5 pigs were re-tested 93 days after the injection of the emulsion. This time they reacted to a 1:20 dilution of OT prepared on synthetic medium. The reactions ranged from ill-defined pinkishness and elevation 10 × 6 mm to well-defined pinkishness and elevation 18 mm in diameter with slight blanching at 24 hours, and at 48 hours were negative to well-defined pink and elevation 16 mm in diameter. Two days later the injection of OT (prepared on broth) in 1:20 dilution resulted in similar reactions. With a dilution of 1:100 at 24 hours the reactions to OT ranged from faint pinkishness and elevation 10 mm to well-defined pinkishness and elevation 13 mm in diameter. These reactions were much weaker at 48 hours. The reactions to 1:1000 OT were doubtful after 24 and negative after 48 hours.‡

Killed *N. asteroides* in water-in-oil emulsion produces a cellular reaction in the subcutaneous tissue which is similar to that found with mycobacteria.[6] It seems, however, that giant cells are less numerous and more of the foreign body type, and the granulation tissue with *N. asteroides* is richer in blood vessels. Like killed mycobacteria, killed *N. asteroides* in water-in-oil emulsion produces a delayed type of specific sensitization. In addition *N. asteroides* sensitizes to tuberculin. However, killed *M. tuberculosis* in paraffin oil sensitizes to very high dilutions of tuberculin up to 1 in 10,000 whereas *N. asteroides* in paraffin oil produces only a slight sensitization to tuberculin. The potentiating effect of *M. tuberculosis* and *N. asteroides* may depend on the cellular reaction these organisms produce. The slight degree of sensitization to tuberculin by *N. asteroides* might be incidental.

Experiments on the effect of *N. asteroides* on the production of antibodies against *S. typhosa* indicate that this organism, unlike mycobacteria, does not enhance agglutinin formation when combined with typhoid vaccine in oil emulsion.

Summary. 1) Killed *N. asteroides* can replace mycobacteria as an adjuvant in the production of (1) skin sensitization to picryl chloride, and (2) isoallergic meningo-encephalitis. 2) Killed *N. asteroides* in water-in-oil emulsion induces a delayed type of sensitization to an extract from this fungus. 3) Killed *N. asteroides* in water-in-oil emulsion sensitizes to tuberculin. The degree of sensitization is slight when compared with that produced by killed *M. tuberculosis* in oil.

SUBSEQUENT NOTE: Demyelination, conspicuous about blood vessels in the CNS, was demonstrated by Spielmeyer's stain. It was found in paralyzed guinea pigs using *N. asteroides* or *M. tuberculosis* or *M. butyricum* as potentiating agents.

---

‡ Skin tests with commercial streptomycin preparations were negative.

Advice received from Dr. L. Raymond Morrison, Department of Neuropathology, Massachusetts General Hospital, is gratefully acknowledged.

16491 P

Effect of Urethane, X-rays, Potassium Arsenite, Benzol, on Survival Time in Transplanted Mouse Leukemia.*

ARTHUR KIRSCHBAUM, SISTER TERESITA JUDD, CHEN SHAN LU,† RUBY M. ENGSTROM, AND HARRY MIXER.

From the Departments of Anatomy and Radiology, University of Minnesota Medical School, Minneapolis.

Urethane depresses the white blood cell count and decreases the size of infiltrated organs in certain cases of human leukemia.[1] Clinical improvement was more striking in chronic myeloid than in chronic lymphatic and acute leukemia.[2] Certain transplanted and spontaneous rodent leukemias were susceptible to the effects of urethane.[3-6]

In these experiments 5 transfer lines of mouse leukemia were studied. Four originated in the high leukemia F strain[7] and a fifth (line RE) was transplanted originally from a spontaneous case which arose in an F_1 hybrid mouse between strains F and NH. Three of these lines of leukemia (15, 686, and RE) were myeloid, and 2 (86 and 291) were lymphoid. The routine method of transfer was by intraperitoneal inoculation of 5,000,000 splenic cells suspended in isotonic saline. Inoculated mice were all F_1 hybrids (strain F $\times$ strain NH) approximately 6 weeks of age.

The methods of treatment with urethane were the following:

a) At the time of transfer an anesthetic dose was administered subcutaneously with no further treatment, or

b) 2, 3 or 4 anesthetic doses were given daily for the first 2, 3 or 4 days. No further treatment was given to these mice, or

c) one-third or one-fifth of an anesthetic dose (1 mg/g) was given daily from the time of inoculation, or

d) after inoculated mice had become leukemic, 2 daily anesthetic doses were given, these being followed by daily doses of 0.33 mg/g body weight.

Survival was significantly prolonged in transplanted myeloid leukemia by all methods of treatment with urethane except the single anesthetic dose at the time of transfer, and 2 doses in the first 2 days following transfer (see Table I); in 2 instances 2 doses increased survival.

Line 291 was a relatively chronic lymphatic leukemia. Although the white blood cell count was depressed by urethane, survival was not appreciably prolonged by the same type of treatment effective for the lines of myeloid leukemia. Lymphoid line 86 responded similarly to line 291 (see Table I for data).

---

* This investigation has been aided by grants from the Donner Foundation, the Jane Coffin Childs Memorial Fund for Medical Research, the National Cancer Institute, and the Graduate Medical Research Fund of the University of Minnesota.

† Fellow of the China Medical Board.

[1] Paterson, E., Haddow, A., ApThomas, I., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.

[2] Hirschboeck, J. S., Lindert, M. C. F., Chase, J., and Calvy, T. L., *J. A. M. A.*, 1948, **136**, 90.

[3] Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

[4] Weir, D. R., and Heinle, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 268.

[5] Law, L. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 158.

[6] Murphy, J. B., and Sturm, E., *Science*, 1946, **104**, 427.

[7] Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1939, **37**, 400.

TABLE I.
Data on the Effect of Urethane on Five Transfer Lines of Leukemia.

Line of leukemia and transfer generation	No. of mice	Daily* urethane, mg/g	Daily inj.	Days after transfer	Avg survival in days (after transfer)
T_3 Line 686-Myeloid	3	0	—	—	34.3 ± .4
	4	1	4	First 4	46-120+†
T_4 ” ” ”	3	0	—	—	37.0 ± 1.4
	2	§	24	28-48	49.0 ± 0.0
	5	1	4	First 2, 8-10	50-95+†
T_5 ” ” ”	5	0	—	—	32.5 ± 1.5
	3	1	1	First day	34.0 ± 1.0
	3	1	2	First 2	41.0 ± 1.4
	3	1	3	” 3	50.0 ± 8.9
	3	1	4	” 4	42-66+‡
	4	§	20	25-45	47.2 ± 1.2
	6	.33	28	First 28	46-66+†
T_5 ” ” ”	3	0	—	—	29.6 ± 1.6
	2	1	1	First day	41.0 ± 4.0
	2	1	2	First 2	30-64+†
	2	1	3	” 3	64+‡
	2	1	4	” 4	51-64+†
	4	.33	25	” 24	47.7 ± 6.3
	3	§	17	26-40	37.3 ± 2.4
T_{11} ” 15 ”	5	0	—	—	25.6 ± 2.0
	3	1	1	First day	28.0 ± 1.4
	3	1	2	First 2	31.3 ± 3.8
	3	1	3	” 3	41-57+†
	3	1	4	” 4	51-57+‡
	3	§	17	18-35	36.0 ± 2.4
T_{10} ” 15 ”	6	0	—	—	26.0 ± 2.0
	6	.33	22	First 25	83.1 ± 15.5
T_{18} ” RE ”	6	0	—	—	18.8 ± .8
	5	.33	29	First 29	31.0 ± 2.4
T_5 ” 291-Lymphoid	6	0	—	—	25.3 ± 6.8
	3	1	1	First day	22.6 ± 4.1
	3	1	2	First 2	20.6 ± .9
	3	1	3	” 3	20.6 ± 4.0
	3	1	4	” 4	29.3 ± 6.1
	6	§	9	15-23	24.8 ± 2.4
	6	.33	23	First 26	26.6 ± 1.9
T_7 ” 86 ”	4	0	—	—	15.7 ± 1.6
	5	.33	18	First 18	21.0 ± 0.0
	5	.2	16	” 16	18.8 ± 2.2

* Aqueous solution of urethane. Except where indicated in text injections were intraperitoneal. The dose per gram of mouse was contained in 0.1 cc distilled water.

† One animal living.

‡ Two animals living.

§ Combined—Daily anesthetic doses for 2 days followed by daily maintenance doses of 0.33 mg/g.

Survival time was increased in myeloid leukemia when treatment was begun even after white blood cell counts were elevated sufficiently to indicate systemic leukemia. However, if white blood cell counts were extremely high and anemia severe, urethane was ineffective in favorably altering the clinical course of the transplanted myeloid disease.

The urethane-treated mice were all growing animals (15-20 g), and failed to gain in weight. However, when growth was suppressed to a greater degree (in mice inoculated

TABLE II.
Comparative Effects of Several Agents on Survival Time in Transplanted Leukemia.

Agent	Daily dose	Line of leukemia	No. of mice	Survival in days
Urethane	0.1 mg/g B.W.*	T_{13}-Line 15-Myeloid	5	38-50
”	0.33 ” ”	”	5	44-67
Benzol (oral)	10 mg	”	5	30-39
Potassium arsenite	0.1 ” (total)	”	4	87-103+†
X-rays (whole body)	50 r	”	5	30-41
None—Controls		”	5	20-24
Urethane	0.33 mg/g B.W.	T_8-Line 686-Myeloid	3	37-56
Benzol (oral)	10 mg	”	3	30-41
Potassium arsenite	0.1 ” (total)	”	3	75-94+‡
X-rays (whole body)	50 r	”	5	36-42
None—Controls		”	4	30-34
Urethane	0.33 mg/g B.W.	T_{23}-Line RE-Myeloid	2	20-27
Benzol (oral)	10 mg	”	2	17-22
Potassium arsenite	0.1 ” (total)	”	2	36-42
None—Controls		”	4	14-18
Urethane	0.33 mg/g B.W.	T_{10}-Line 86-Lymphoid	3	17-21
Benzol (oral)	10 mg	”	3	15-17
Potassium arsenite	0.1 ” (total)	”	3	21-25
None—Controls		”	4	11-17
Urethane	0.1 mg/g B.W.	T_8-Line 291-Lymphoid	4	21-25
”	0.33 ” ”	”	4	25-29
Benzol (oral)	10 mg	”	5	21-26
Potassium arsenite	0.1 ” (total)	”	4	22-28
None—Controls		”	5	20-21

* B.W. = Body Weight.
† 3 mice still living.
‡ 2 mice still living.

with line 15 leukemia) by feeding a diet containing 5% p-aminobenzoic acid, survival time was not prolonged.

Other agents (x-rays, benzol, potassium arsenite, carcinogenic hydrocarbons, colchicine) may increase survival time in certain lines of transplanted leukemia and lymphosarcoma.[8–10] The effects of x-rays, benzol, potassium arsenite, and urethane were compared, treatment with all agents being started at the time of transfer. Results are given in Table II.

Of the agents tested, potassium arsenite was the most effective in increasing survival time in the 3 transfer lines of myeloid leukemia, although it had little if any effect on the course of the lymphoid disease. Treatment with either benzol or x-rays also prolonged survival time in myeloid leukemia (Table II). Mice gained in weight during treatment with either potassium arsenite or benzol, whereas animals treated with urethane and x-rays were smaller (15 g and 17 g as contrasted with 23 g). All mice were of approximately the same weight at the time of transfer of leukemia.

As a result of passage through successive generations of hosts, leukemic cells are altered[11] so that results achieved with transplanted leukemia may not apply to the spontaneous disease. Apparently the efficacy of a particular compound may be determined by the type of leukemia, whether myeloid or lymphoid, and genetic differences in leukemias of the same type. Early transfer generations, where survival is relatively long, are probably more favorable for testing than the late trans-

---

[8] Flory, C. M., *A.A.A.S. Research Conference on Cancer*, 1945, p. 291.

[9] Stamer, S., and Engelbreth-Holm, J., *Acta Path. et Microbiol. Scand.*, 1943, **20**, 360.

[10] Lits, F. J., Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1938, **34**, 196.

[11] MacDowell, E. C., Potter, J. S., and Taylor, M. J., *Proc. Nat. Acad. Sci.*, 1939, **25**, 416.

fer generations of lines which have become highly malignant as a result of numerous transfers. A variety of lines should be tested with agents offering encouraging results on particular lines.

Summary. Survival time was lengthened significantly in 3 transfer lines of mouse myeloid leukemia by treatment with either potassium arsenite, urethane, x-rays, or benzol in decreasing order of effectiveness. Two transfer lines of lymphoid leukemia grown in genetically identical hosts did not respond favorably to the same treatment with the chemical agents. Potassium arsenite was more effective than urethane on a weight basis, and in the effective dose range was tolerated better than urethane.

16492

Further Experiments on Bridging of Long Nerve Gaps in Monkeys.*

EBEN ALEXANDER, JR., ROBERT P. WOODS, PAUL WEISS.

From the Children's Hospital, Department of Surgery, Harvard Medical School, and the Department of Zoology, University of Chicago.

The method of bridging gaps in peripheral nerves by a fiber cable embedded in a tubulated blood bridge, as described by Weiss and Taylor[1] for lower mammals has been successfully extended to monkeys by Matson, Alexander and Weiss.[2] The nerve gaps in these experiments measured up to 14 mm in length. The present experiments were undertaken to determine the feasibility of bridging longer gaps (from 22 to 32 mm), by the same technic.

Experiments. Five adult *Macaca rhesus* monkeys were used. The technic was essentially the same as in the earlier experiments. A section of the tibial nerve was cut out and the stumps allowed to retract; a segment of internal jugular vein was threaded over the proximal stump; the desired length of the gap was fixed by a tantalum sling stitch without tension; the nerve stumps were connected axially by a cable of finest nylon fibers (.006 inch); a trough made from polyethylene tubing of nerve caliber was placed under the cable-spanned gap and filled with blood from the jugular vein; after firm clotting had occurred, the trough was removed; finally, the sleeve of the vein was pulled over the bridged gap and secured to each nerve stump by a silk stitch. After wound closure with silk, no further provisions were made for the protection of the site of operation. The animals were set free in large cages. All remained in good health.

No detailed records were kept of the course of nerve recovery, and all tests were left to the terminal exploration, which was made under intravenous nembutal anesthesia, 289 to 333 days after the operation. After measuring both calves and exposing the nerve, the responses of leg and foot muscles to faradic stimulation of the nerve were determined. Segments of the regenerated nerve and of the control nerve from the opposite side were removed for histological study. In 4 of the 5 cases, the weights of the gastrocnemius muscles of both sides were also recorded.

Results. There were no technical failures in this series. Wounds healed without infection. In no case had the activities of the animals during the early postoperative period disrupted the nerve bridge. On re-exploration, the nerve bridge, after being freed from moderate adhesions, presented itself as an elon-

---

* This work has been aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Weiss, Paul, and Taylor, A. C., *J. Neurosurg.*, 1946, **3**, 375.

[2] Matson, D. D., Alexander, Eben, Jr., and Weiss, Paul, *J. Neurosurg.*, 1948, in press.

gated swelling of the nerve 1½ to 2 times the diameter of the normal nerve.

The degrees of recovery in the various cases are summarized in Table I. To gross observation, the functional use of the limbs appeared recuperated, but no detailed study of coordination has been attempted. The electrical stimulation of the exposed tibial nerve above, as well as below, the anastomotic site, yielded good contractions in the gastrocnemius muscles, the posterior tibial muscles and the long flexor muscles of the toes in all animals. The intrinsic muscles of the foot, studied after severance of the Achilles tendon, showed no perceptible response in two cases, fibrillary twitching in two others, and a marked response, with clear-cut opposing motion of the great toe, in the fifth.

Weight recovery in the reinnervated muscles was likewise very satisfactory. While denervated muscles are reduced to 30% of control weight (88 days after transection of the tibial),[2] the calf muscles innervated by the bridged nerves have recovered 77-95% of their controls, with an average of 85% (Table I). The degree of recovery expressed in these figures is actually higher than that observed in the previous series with shorter gaps (50-93%, with an average of 63%), but this difference may be attributable to the longer recovery period in the present series (289-333 days, as compared to 222-288 days). It is evident that the increase in the length of the gap has not reduced the effectiveness of the bridge in facilitating nerve regeneration in this series of experiments.

Histological observations. Sections through corresponding levels in midcalf of the regenerated and control tibial nerves, treated with osmic acid, fully confirm the results of the earlier series with shorter gaps. The regenerated distal nerve stumps contain approximately their normal quota of fibers. An actual count (monkey 74) showed 10,460 fibers in the regenerated nerve, and 11,565 fibers in the opposite control nerve. Numerically speaking, the volume of innervation has thus been practically fully restored. This point deserves emphasis since one encounters reports of successful nerve regeneration containing histolog-

TABLE I.
Recovery of Muscles After Nerve Regeneration Over Long Nerve Gaps with Bridges.

No. of monkey	Length of gap, mm	Days post-op.	Atrophy* of calf, %	Response to faradic stimulation of nerve				% of wt recovery in Gastrocnemius
				Gastrocnemius	Posterior tibial	Long flexors	Intrinsic foot muscles	
66	32	333		+	+	+	0	(Control muscle unavailable)
72	23	321	6	++	++	++	0	82
73	25	302	13	++	++	+	±	86
74	26	289	5	+	+	++	+	77
75	22	307	5	++	++	++	±	95

* Percentage difference of circumference between calves on reinnervated and opposite control sides.

ical pictures of regenerated fibers in the distal stump, but without substantiation of their number.

As in the former series, the diameters of the regenerated branches were subnormal, with only a few reaching the 8-9 micra class. This caliber deficit indicates that the fibers have been encroached upon at some proximal level by endoneural fibrosis, possibly at the points where the nylon cable was sewn through the stumps. It has been shown that the narrowing of the lumen of a fiber at any one level limits the caliber growth of the distal portion of the fiber.[3,4] While one must suspect that permanently subnormal calibers entail some deficiencies in function,[5] these have not been severe enough to become grossly discernible.[2]

Conclusions. Comparing these results with those of the earlier series involving gaps of about half the length, no evidence of a decline of the power of regeneration with the increase in the length of the gap can be detected. It may be reasonable to assume that the gap could be lengthened even more without becoming unbridgeable.

Summary. Gaps of from 22-32 mm in length in tibial nerves of monkeys were closed by nylon cables embedded in tubulated blood bridges.[1,2] The reinnervated muscles regained an average of 85% of their normal weight. The distal regenerated nerve fibers were approximately normal in number, though subnormal in size. There has been no reduction of regenerative power with increased length of the gap in this series as compared to a previous series with shorter gaps.

---

[3] Weiss, Paul, and Taylor, A. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 77.

[4] Weiss, Paul, and Hiscoe, Helen B., *J. Exp. Zool.*, 1948, **107**, 315.

[5] Young, John Z., *Physiol. Rev.*, 1942, **22**, 318.

16493

Erythrophagocytosis in Bone Marrow Culture with Relation to Antagonism of Pteroylglutamic Acid.*

HERBERT SALIS.† (Introduced by Arnold D. Welch.)

From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio.

A small series of cultures of bone marrow has been studied, as part of a larger investigation of the function of pteroylglutamic acid (PGA).[1] Through the addition of PGA-antagonists [(crude antagonist N67-A,[2-4] 'Methopterin' (a methyl pteroylglutamic acid of unidentified structure,[5]) and 'Aminopterin' (N–[–{[(2,4-diamino-6-pteridyl) methyl]-amino}-benzoyl]-glutamic acid][6‡] it had been hoped to achieve *in vitro* a maturation arrest

---

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

This paper is published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

† Resident, Department of Medicine, Crile Veterans Administration Hospital, Cleveland, Ohio. The author is indebted to Dr. Robert W. Heinle and Dr. Arnold D. Welch for their valuable aid and advice.

[1] Welch, A. D., Heinle, R. W., Pritchard, J. A., and Salis, H., *Fed. Proc.*, 1948, **7**, 300.

[2] Welch, A. D., Heinle, R. W., Sharpe, G., George, W., and Epstein, M., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 364.

[3] Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 368.

[4] Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

[5] Hultquist, M. E., and co-workers, unpublished data.

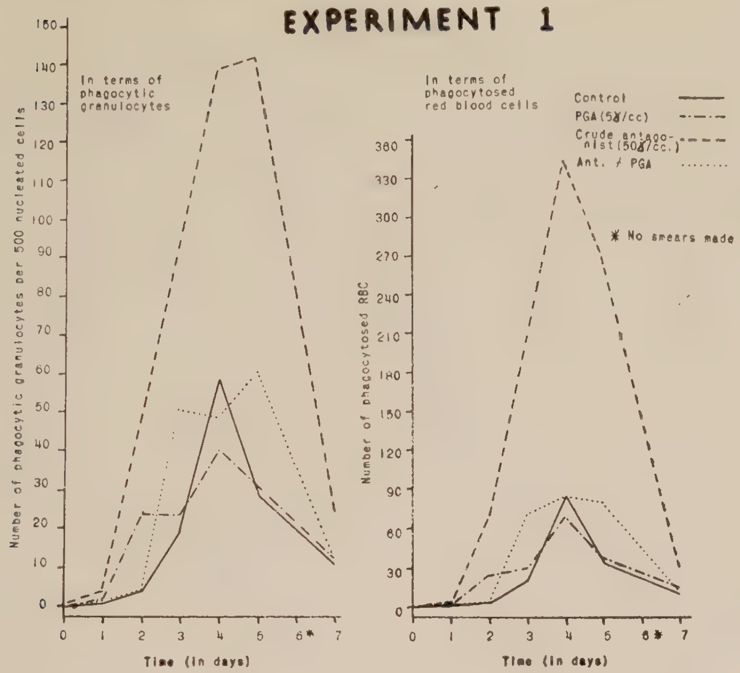

EXPERIMENT 1

FIG. 1.

Erythrophagocytic effects of crude PGA-antagonist (N67-A) and its reversal by PGA.

in the erythroid series. Although this did not develop, extensive phagocytosis of erythrocytes, chiefly by granulocytes, was encountered, with a definite relationship to the presence of the PGA-antagonist. Because this phenomenon may have clinical implications, a summary of the results is being reported.

The culture method utilized was essentially that of Osgood and Brownlee.[7] Human umbilical cord serum in a concentration of 30-35% in a balanced salt solution was employed as the basic medium, in the manner described by these authors. Bone marrow cells were procured by the aspiration of 2 to 7 cc of marrow content from the sterna of patients without demonstrable hematologic disorders.

Observations in 2 experiments are shown in the accompanying graphs. A third experiment, involving the use of 'Aminopterin' (55 μg/cc), revealed, on the fifth and last day of the experiment, a count of 16 phagocytic granulocytes per 200 nucleated cells in the culture containing 'Aminopterin,' as opposed to 2 such cells in the control culture.

Except for an occasional eosinophil and several myelocytes, the phagocytic cells were either segmented or band-form polymorphonuclear neutrophils. Large macrophages, similar to those usually described, were found in all cultures after the first to third day, but these cells were only slightly phagocytic of erythrocytes, and were not included in the category of erythrophagocytic cells. (These macrophages have persisted in culture for more than 70 days). Only phagocytosed erythrocytes with distinct morphology were enumerated, thereby excluding cells in an advanced state of digestion. As many as 10 or 11 red cells have been counted in a single phagocyte. Occasionally, a normoblast was found phagocytosed.

From examination of the graphs, it will be noted that (a) the number of erythrophagocytic granulocytes in the culture containing a

---

[6] Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

‡ The antagonists of pteroylglutamic acid were supplied by the Lederle Laboratories Division, American Cyanamid Company.

[7] Osgood, E. E., and Brownlee, I. E., *J.A.M.A.*, 1937, **108**, 1793.

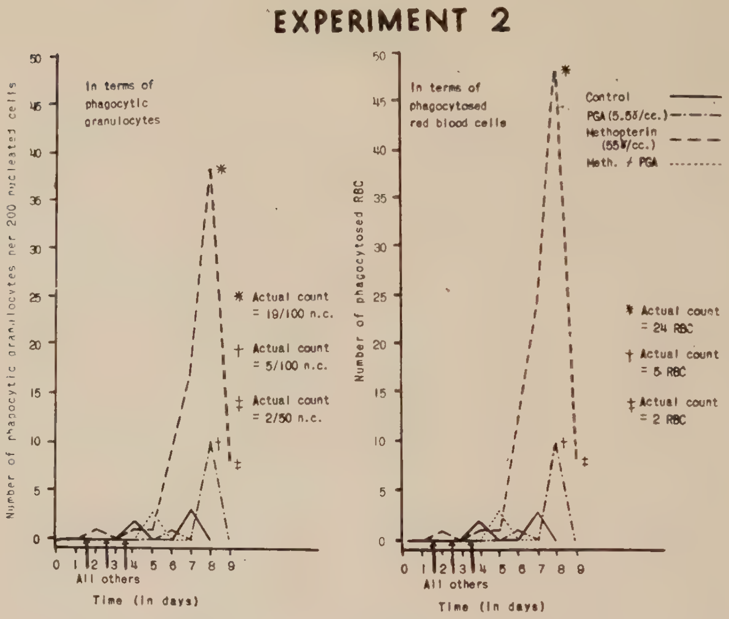


FIG. 2.
Erythrophagocytic effects of "Methopterin" and its reversal by PGA. Arrows indicate solid line representing all cultures other than those shown above this line.

PGA-antagonist became much greater than that of any other culture, (b) these cells, in the cultures containing antagonist, ingested more erythrocytes than those in the other cultures, and (c) these effects were prevented by the addition of PGA.

Daily leucocyte and differential counts of 200 nucleated cells revealed the relative and absolute numbers of segmented and band-form polymorphonuclear leucocytes in the PGA-antagonist cultures to be similar to, or often less than, those in the other cultures. Thus, the greater erythrophagocytosis prevailing in the antagonist cultures was not due to the presence of a greater number of potentially phagocytic cells.

These findings are of interest because of the occurrence of erythrophagocytosis in certain cases of marked anemias. Such phagocytosis has been observed in the peripheral blood in idiopathic aplastic anemia,[8] pernicious anemia,[9-11] acute hemolytic anemia of the Lederer type,[8,12] severe "secondary anemia,"[13] sickle-cell anemia (more pronounced in the liver, spleen, and lymph nodes),[8,14-17] erythroblastosis fetalis,[8,11,18] paroxysmal hemoglobinuria,[11] and in marked anemic states associated with leukemias,[11,19] parasitic infections (especially malaria),[11] and toxic or infectious states (especially subacute bacterial endocarditis).[11] Oroya fever, with a severe anemia resembling pernicious anemia, evidences marked erythrophagocytosis in the bone marrow, spleen and liver.[20,21] The anemia of Hodgkin's disease has been ascribed to erythrophagocytosis (in lymph nodes, liver, lungs and bone marrow),[22] and the severe anemia of primary splenic panhematopenia has been attributed to excessive phagocytic function of the spleen.[23] Throughout the literature, macrophages have been the chief phagocytic cell reported. Only in patients with severe pernicious anemia,[9] and a severe "secondary anemia" (probably of a leukemia)[13] have granulocytes been the predominating phagocytic cell type.

---

[8] Whitby, L. E. H., and Britton, C. J. C., *Disorders of the Blood*, 5th Ed., The Blakiston Co., Philadelphia, 1946, p. 381.

[9] Hopkins, J. G., *Arch. Int. Med.*, 1910, **6**, 270.

[10] Kaznelson, P., *Deutsches Arch. f. klin. med.*, 1918, **128**, 131.

[11] Abt, A. F., *Am. J. Dis. Child.*, 1931, **42**, 1364.

[12] Hargraves, M. M., Herrell, W. E., and Pearman, R. O., *Proc. Mayo Clinic*, 1941, **16**, 107.

[13] Rowley, M. W., *J. Exp. Med.*, 1908, **10**, 78.

[14] Sydenstricker, V. P., *J.A.M.A.*, 1924, **83**, 12.

[15] Diggs, L. W., *South. Med. J.*, 1932, **25**, 615.

[16] Mason, V. R., in Downey, H., *Handbook of Hematology*, Paul B. Hoeber, Inc., New York, 1938, pp. 2343-44.

[17] Stasney, J., *Am. J. Path.*, 1943, **19**, 225.

[18] Wyatt, I. C., Cooper, M. B., and Groat, W. A., *Am. J. Dis. Child.*, 1938, **56**, 1319.

[19] Bowcock, H., and Bishop, E. L., *Ann. Int. Med.*, 1930, **3**, 1252.

In animals, toxic or infectious states have led to anemic conditions associated with erythrophagocytosis.[24] Most prominent of these is the bartonellosis of rats,[25] similar to Oroya fever in man. It is of interest that this condition is reported to be improved by treatment with PGA.[26] Other studies in man and rodents indicate an increased phagocytic function of granulocytes in anemia (including pernicious anemia), the increase in phagocytosis being roughly proportional to the severity of the anemia.[27]

The clinical and experimental data suggest, therefore, that erythrophagocytosis may be associated with a PGA-deficient state. Further investigation is required to ascertain if such a relationship exists.

Summary. Blood cell cultures grown in the presence of PGA-antagonists exhibited marked erythrophagocytosis by granulocytes when compared with cultures containing no antagonist or containing antagonist and PGA. It is suggested that PGA-deficiency might account for erythrophagocytosis observed in certain instances of anemia in humans and animals.

---

[20] *Report of First Expedition to South America,* 1915, Cambridge, Harvard University Press, pp. 50-51.

[21] Ash, J. E., and Spitz, S., *Pathology of Tropical Diseases,* W. B. Saunders Co., Philadelphia, 1945, pp. 25-26.

[22] Marschal, G., Mahoudeau, D., Loc'h Le, and Brun, C., *Bull. et mem. Soc. d'hop de Paris,* 1941, **57**, 15.

[23] Doan, C. A., and Wright, C., *Blood,* 1946, **1**, 10.

[24] Jaffe, R. H., in Downey, H., *Handbook of Hematology,* Paul B. Hoeber, Inc., New York, 1938, p. 1017.

[25] Weinman, D., *J. Infect. Dis.,* 1938, **63**, 1.

[26] Smith, H. M., and Emery, F. E., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 178.

[27] (a) Berry, L. J., Leyendecker, R. M., and Spies, T. D., Morphologic Hematology, Special Issue No. 1 of *Blood,* Grune and Stratton, New York, 1947, p. 98; (b) Berry, L. J., and Haller, E. C., *ibid.,* p. 108; (c) Berry, L. J., and Haller, E. C., *ibid.,* p. 117.

16494

Electrokinetic Change in Human Erythrocytes During Adsorption and Elution of PR8 Influenza Virus.*

MARTIN HANIG. (Introduced by Thomas Francis, Jr.)

From the Department of Epidemiology and the Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Mich.

Hirst[1,2] and McClelland and Hare[3] found that PR8 strain of influenza virus could be adsorbed by chicken erythrocytes with their accompanying agglutination. On standing it was also found that the virus was gradually eluted from the cells and that the cells could no longer adsorb subsequent additions of that

---

* Aided by grants from the National Foundation for Infantile Paralysis, Inc., and from the Commission on Influenza, Army Epidemiological Board, Office of Surgeon General, U. S. Army.

[1] Hirst, G. K., *Science,* 1941, **94**, 22.

[2] Hirst, G. K., *J. Exp. Med.,* 1942, **76**, 195.

[3] McClelland, L., and Hare, R., *Can. Pub. Health J.,* 1941, **32**, 530.

virus. The agglutination phenomenon was rapidly developed into a quantitative test[4–6] for influenza viruses and became an invaluable tool in the elucidation of their properties and in their purification.[7–9] Little is known concerning the physical or chemical mechanism of the agglutination, however, and the present work is the initial aspect of such an investigation.

Since no detectable change in the electrokinetic characteristics of red blood cells in the presence of egg albumen, gelatin, casein, hemoglobin and fibrinogen has been observed, it has been concluded that the cell retains its surface properties even in the presence of large amounts of proteins.[10] The present paper, however, reports observations that the electrokinetic potential of human erythrocytes is greatly decreased by minute additions of influenza virus, that this decrease accompanies the adsorption and elution phenomena, and that the reaction is dependent upon the concentration of added virus.

Materials and methods. Preparation of virus. The PR8 strain of Type A influenza virus maintained by egg passage was employed. Twenty-five dozen 10-day embryonated hen's eggs were inoculated by the allantoic route with 0.1 ml of infectious allantoic fluid diluted to 10^{-3} with 0.9% NaCl. The inoculum before dilution had an agglutination titer of 1-5120 by a pattern test. The eggs were sealed with paraffin and were incubated at 36°C for 48 hours. The allantoic fluids were harvested by pouring through a funnel lightly plugged with glass wool. The resulting 1900 ml of infectious allantoic fluid had an agglutination titer of 1-5120. Adult chicken red blood cells were added to make a 2% suspension, and adsorption was allowed to take place for 2 hours at 4°C. The cells were centrifuged out of the suspension and were resuspended in 80 ml M/15 sodium potassium phosphate buffer, pH 7.4,[11] at 37°C for 90 minutes for elution to take place. The cells were removed and washed by centrifugation with 50 ml buffer at 37°C. The supernates were combined and clarified at 3000 r.p.m. for 20 minutes. The virus was sedimented from this solution in a high speed angle centrifuge for 90 minutes at 14,400 r.p.m. at 4°C. The resulting pellets were dissolved in 900 ml buffer at 4°C. Fresh chicken erythrocytes were added to make a 2% suspension and an identical cycle of adsorption, elution and differential centrifugation was repeated. The total yield was 12.0 ml of virus preparation[†] having a titer of 1-160,000 and containing 2.8 mg of material per ml when dried in air for 3 hours at 110°C and corrected for the salt content. The concentrated virus material when coated on collodion particles in M/15 sodium potassium phosphate buffer, pH 7.4 had an electrophoretic mobility of 0.67 μ/sec./volt./ cm at a concentration of 0.14% at 22.5 ± 1.5°C. Electron micrographs showed little evidence of extraneous material or particles of smaller size, the purification procedure having been designed to reduce the amount of normal, acidic component.

Titration of virus. The titrations were carried out by the procedure of Salk,[6] employing a suspension of 0.25% chicken red blood cells, with the exception of the end point taken. The modification used served to decrease the great differences in reading end points inherent in the 2-fold dilution procedure. The readings

---

[4] Hirst, G. H., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.

[5] Miller, G., and Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 185.

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

[7] Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80**, 531.

[8] Sharp, D. G., Taylor, A. R., McLean, I. W., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

[9] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

[10] Abramson, H. A., Moyer, L. S., and Gorin, M. H., *Electrophoresis of Proteins*, Rheinhold Pub. Corp., N. Y., 1942, 307.

[11] Clark, W. M., *The Determination of Hydrogen Ions*, The Williams and Wilkins Co., Baltimore, 1928, 212.

[†] Hereinafter the term, virus, refers to this concentrated material containing the PR8 strain of influenza virus, Type A, and the quantitative values refer to the dry weight of the preparation.

TABLE I.

Relation Between Mobility of Erythrocytes and Hemagglutination Titer with Different Concentrations of Virus.

16.9 µg/ml			3.11 µg/ml			0.933 µg/ml			0.350 µg/ml			0.311 µg/ml		
Min.	Mobility	Titer	Min.	Mobility	Titer	Min.	Mobility	Titer	Min.	Mobility	Titer	Min.	Mobility	Titer
							Controls.							
—33		960	—26		160	—21		64	—19	1.28		—22		10
—10	1.34		—10	1.33		— 8	1.38		— 9		12	—17	1.37	
235	1.28		1391	1.28		15	1.35		51	1.30		19	1.34	
375	1.30					155	1.31		235	1.30		379	1.31	
1486	1.30					388	1.35		1473	1.35		1445	1.38	
						1719	1.39							
							Virus.							
5	0.96		14		160	10	1.37		20	1.25		12		16
8		480	18	1.25		16		32	29		0	40		2
30		160	36		35	34		8	69		0	45	1.21	
50	0.68		88		10	35	1.37		126	1.19		91	1.28	
60		240	98	1.01		62		8	149		0	104		0
100	0.61		170		20	75	1.19		195	1.17		176		0
184		800	193	0.69		82		8	225		0	184	1.28	
207	0.52		235		50	99		8	313	1.05		244	1.21	
265		1280	267	0.65		134		8	712		0	260		0
314	0.57		291		70	173	1.18		734	0.96		348	1.19	
340		960	364		70	179		8	1380		0	353		0
870	0.49		369	0.56		230	1.03		1390	0.86		389	1.12	
895		1280	385		160	234		8	1583		2	743	1.14	
1422		1120	1361		240	251		16	1656	0.78		763		0
1505	0.50		1431	0.58		282		16	1725		4	1389		0
1681	0.50					364	0.91		1749	0.66		1467	0.99	
						365		16	2785		16	1775	0.60	
						1435		64	2893	0.52		1807		6
						1494	0.66					2873		24
						1688		128						
						1693	0.63							

of +, ±, or O were read as in Salk's method. However, ± instead of + was taken as the end point. Where + proved to be the observed end point followed by O, ± was interpolated halfway between the two values. All titrations were run in duplicate and when the duplicates differed, as they occasionally did, their values were averaged. The dilutions for the titrations were made in the same stock M/15 sodium potassium phosphate buffer, pH 7.4, as were those for the electrophoretic experiments.

Microelectrophoretic measurements. The electrophoretic mobility was measured by the method of Moyer.[12] Human type O red blood cells rather than chicken red blood cells were used because of their smaller size and lower sedimentation rate. They were prepared by washing 3 times with M/15 sodium potassium phosphate buffer, pH 7.4 and were diluted to 10% suspensions in the buffer immediately after their preparation. They were stored at 4°C. The erythrocytes were never used after 7 days although they maintained their original electrokinetic properties at least until 10 days after shedding. In one experiment in the presence of 5.6 μg of virus suspension per ml there was no significant electrophoretic difference between type O and type AB cells. Therefore, type O cells from a single donor were used throughout. Agglutination of the erythrocytes into groups of 2-6 in the presence of the virus could regularly be seen in the microscope during the measurements. These groups did not seem to differ in mobility from single cells and were also used in measuring mobility. Equal numbers of 12-15 measurements in each direction were made in the microelectrophoresis cell at the 0.21 level. These were averaged. The erythrocytes were suspended at a concentration of 0.25% in buffer. The measurements were conducted at 22.5° ± 1.5°C. The instrument was rigorously cleaned with dichromate cleaning solution and distilled water before the introduction of each sample.

Experimental. Two 250 ml flasks were used to contain the control and the virus-red blood cell suspensions for the duration of a single experiment. That one serving as the control flask contained 100 ml of a 0.25% suspension of human type O erythrocytes in buffer. The second flask contained 150 ml of purified virus preparation in the selected dilution with the same stock buffer for the particular series of observations. Just prior to the addition of the erythrocytes to the virus suspension, a 3.75 ml sample was withdrawn for subsequent titration. The same volume of the 10% stock suspension of red blood cells was then added to the virus suspension and the time was noted. The time of admixture of the reagents is considered to be zero time for comparison of the different runs at the various concentrations. Measurements of agglutinating activity of the virus and of electrophoretic mobility of the red cells which served as controls, and which were made before zero time are tabulated in negative time increments, Table I. At convenient intervals after the admixture of reagents, microelectrophoretic measurements were made on the virus and control samples. These measurements usually required 10-15 minutes. The average time between the first and last readings was selected as the time of the measurement from zero time.

Concurrent with the microelectrophoretic measurements 3.0 ml samples of the virus-erythrocyte suspensions were periodically withdrawn for titration. These were immediately centrifuged at 2,500 r.p.m. for 10 minutes to remove the erythrocytes. The supernates were carefully withdrawn from the red cell precipitates, and the time was noted. The supernates were stoppered and placed in the refrigerator at 4°C for subsequent titration. Upon completion of a run, usually within 1800 minutes from zero time, all the supernates and the original virus suspension were titered together under the same conditions with one pool of chicken erythrocytes.

Results. The relation between the quantity of virus found in the supernates during the adsorption and elution processes and the electrophoretic mobility of the erythrocytes at varying time intervals is shown in Table I and Figs. 1, 2, 3. The concentrations of the virus material were 16.9, 3.11, 0.933, 0.350 and 0.311 μg per ml respectively. The titers are expressed as dilutions of the stock virus sus-

---

[12] Moyer, L. ., *J. Bact.*, 1936, **31**, 531.

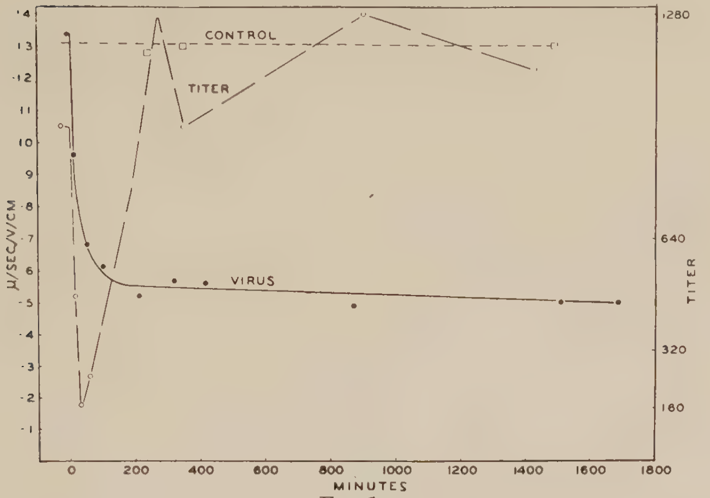


FIG. 1.
Relation between mobility of 0.25% erythrocytes and adsorption-elution in the presence of excess virus, 16.9 μg/ml.

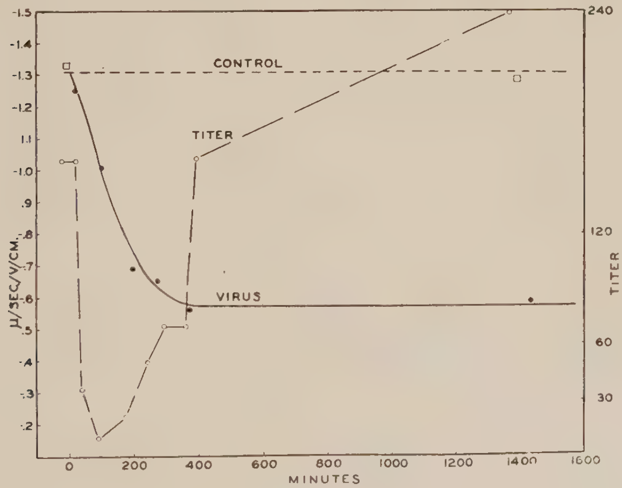


FIG. 2.
Relation between mobility of 0.25% erythrocytes and adsorption-elution in the presence of the optimal concentration of virus, 3.11 μg/ml.

pension containing 2.8 mg/ml.

At the higher virus concentrations, 16.9 μg/ml (Fig. 1, Table I), there is an initial sharp drop in titer as adsorption of virus takes place on the cells. This is followed by rapid elution. Corresponding to the adsorption of the virus by the erythrocytes there is a sharp drop in the electrophoretic mobility of the cells from the normal value of 1.32 to about 0.55 μ/sec/v/cm. When the virus is eluted from the cells, as indicated by the rise in titer of the supernate, there is no corresponding rise in the electrophoretic mobility. It remains at the lower value.

As the virus concentration is gradually decreased down to 0.311 μg/ml, (Figs. 2, 3, Table I), the sharp initial drop in titer persists, but the elution is delayed. The negative slope of electrophoretic mobility also decreases. When a concentration of 0.350 μg/ml is reached (Fig. 3), the virus remains adsorbed to the erythrocytes for as long as 1400 minutes

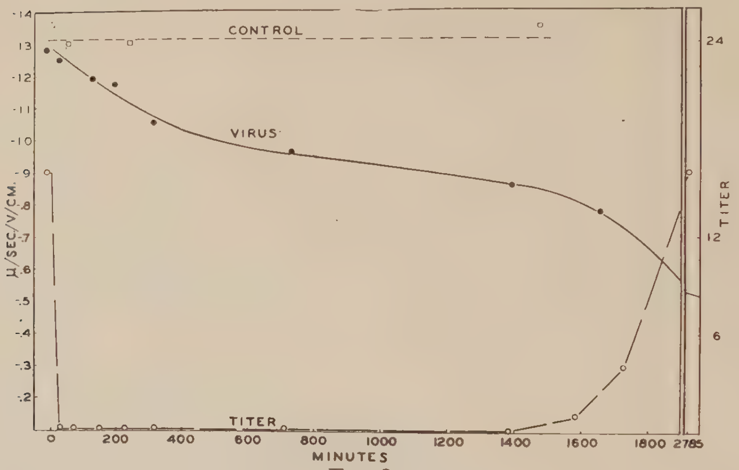


FIG. 3.

Relation between mobility of 0.25% erythrocytes and adsorption-elution in the presence of insufficient virus, 0.350 μg/ml.

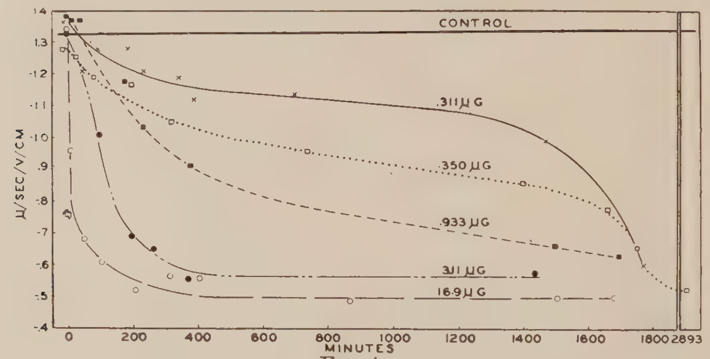


FIG. 4.

Comparison of mobility of 0.25% erythrocytes in the presence of various concentrations of virus illustrating how the mobility pattern changes with the concentration of virus.

before elution is detected, and at this time the electrophoretic mobility also begins to fall to the same low value reached by those cells treated with the higher concentrations of virus. It may be seen, therefore, that the shapes of both the electrophoretic mobility curves and the adsorption-elution curves are not only interdependent but also are concentration dependent. The dependence of adsorption and elution upon virus concentration at constant concentration of erythrocytes is illustrated in Fig. 5. For greater clarity the curve for 0.350 μg virus/ml has been deleted as it falls so close to the curve at 0.311 μg virus/ml. The dependence of electrophoretic mobility upon virus concentration is illustrated in Fig. 4. The heavy line representing the controls, no virus added, is a composite of 19 points taken concurrently with the curves made in the presence of the various concentrations of virus. It was drawn by the method of least squares. The standard deviation, σ, is 0.035.

Discussion. The changing shape of the electrophoretic mobility curves as the virus concentration is increased, (Fig. 4), leads to the concept that there might be an optimal ratio of virus to cells at which all the receptors of the cells are acted upon at the same time. At this concentration it might be expected that for a short interval little or no virus would be found in the supernate. It would rapidly be adsorbed on, and then eluted from, the cells. From the adsorption-elution titrations, Fig. 5, it appears that the optimal virus concentra-

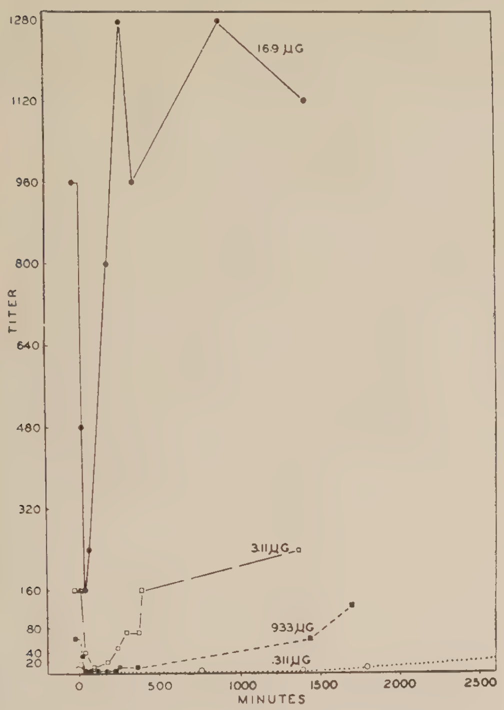


Fig. 5.

Comparison of the supernatant virus titers illustrating the changing pattern of adsorption-elution for various concentrations of virus in contact with 0.25% erythrocytes.

tion in the presence of 0.25% cells would be very close to 3.11 μg virus/ml. At a concentration of 16.9 μg/ml all the virus was not removed from the supernate while at 0.933 μg/ml it remained attached to the cells for over 200 minutes. Using the values, 3.11 μg virus/ml, 40 mμ as the radius of the anhydrous virus,[7] 1.27 as its density,[7] and 5.54×10^6 as the number of erythrocytes per cu mm of whole blood by actual count, it may be calculated that at the point of simultaneous saturation of the cell receptors by the virus, there are 298 PR8 particles to one erythrocyte. If one considers the erythrocyte as a sphere with a surface area of $120\mu^2$,[13] and the virus as a disk with a radius of 40 mμ, it is evident that 23,900 PR8 particles would be required to cover the erythrocyte with just a single layer. According to this calculation at the optimal concentration only 1/80 of the surface area of the cell is actually covered by the virus, a value inconsistent with the possibility of the cell's assuming the charge of a complete layer of the adsorbed virus. To support this evidence it is found that after elution of the virus from the cells, the cells continue to have the same lowered mobility. This mobility is lower than that of the virus itself and indicates that it is the mobility of the altered cells.

The changing shapes of the electrophoretic mobility curves with time for various virus concentrations (Fig. 4) may be interpreted to mean that at low virus concentrations the virus is adsorbed indiscriminately by the individual cells in chance amounts. When virus particles elute they are immediately adsorbed by other receptors on the same or on adjacent erythrocytes, gradually reducing the electrokinetic potential of all the cells in the suspension. As the ratio of virus particles to unreacted receptors on the cells increases, the rate at which the electrophoretic mobility falls off rapidly increases. The same low value of 0.5-0.6 μ/sec/v/cm is eventually reached as was attained 1500 minutes before by those cells in contact with higher virus concentrations.

That the decrease in electrophoretic mobility of the erythrocytes takes place while they are in intimate contact with the virus is indicated in Figs. 1, 2, and 3. Particularly in Fig. 3 where the virus and cells are in contact for a long time, it may be seen that some kind of reaction expressed by the decrease in mobility is in progress, and that this reaction is not complete until elution is complete. These results clearly indicate an ionogenic change in the character of the erythrocyte which is exhibited by its altered electrophoretic mobility. The nature of the ionogenic change, whether it is caused by loss of a charged substance from the cell, change of the spatial configuration of charged groupings of the cell, or adsorption by the cell of charged elements of the solvent after the cell's treatment by virus, is still obscure. Further experiments are in progress to determine a more exact interpretation of the phenomenon.

Aggregation of the virus after high speed centrifugation and disaggregation after adsorption and elution by the erythrocytes are clearly indicated in Fig. 5. It may be seen that for each concentration of virus in the adsorption-elution curves, the titer before adsorption is considerably lower than after elution. That packing of the virus during sedimentation causes aggregation is borne out by the electron micrographs which were made after centrifugation and before adsorption and elution by the erythrocytes. The micrographs showed most of the virus particles in a state of aggregation.

---

[13] Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, The Williams and Wilkins Co., Baltimore, 1943, 11.

Conclusions. The electrophoretic mobility of human type O erythrocytes was found to decrease from 1.32 to 0.5-0.6 μ/sec./v./cm in the presence of 0.311 to 16.9 μg/ml of a highly purified preparation of the PR8 strain of influenza virus.

Adsorption and elution of the virus, and agglutination of the erythrocytes was found to be concurrent with the decrease in electrophoretic mobility of the erythrocytes.

The time of intimate contact of virus with cells and the pattern of decrease of electrophoretic mobility are not only interdependent but also dependent upon the concentration of added virus.

At the optimal ratio of virus to erythrocytes, as determined by the mobility and titration curves, it has been calculated that only 1/80 of the erythrocytic surface is covered by virus particles and that this value corresponds to about 298 PR8 virus particles to one erythrocyte.

16495

Relation of Induced Sulfonamide Resistance to Biochemical Properties in Alpha Streptococci.

WILLIAM E. CLAPPER AND MARY E. HEATHERMAN.
(Introduced by Richard Thompson.)
From the Department of Bacteriology, University of Colorado Medical School, Denver, Colo.

A decrease in the production of hydrogen peroxide accompanying an induced increase in resistance to sulfonamide bacteriostasis was first observed in a strain of pneumococcus by MacLeod.[1] Since some streptococci produce hydrogen peroxide, it seemed desirable to determine whether or not these organisms would produce less peroxide when they were made resistant to sulfonamide, whether other biochemical changes would be produced, and what relation these changes might have to the mechanism of resistance.

Loss of peroxide producing ability by resistant organisms. Five strains of greening streptococci originally obtained from saliva were tested for susceptibility to sulfathiazole by the serial dilution method, and for hydrogen peroxide production by the method of Main and Shinn[2] with slight modifications. They were then made resistant by transferring at intervals of 3 to 5 days in broth containing one-half the inhibiting concentration. All organisms were transferred 15 times. Sulfathiazole susceptibility and hydrogen peroxide production were then determined again. The results of the determination are shown in Table I. The sulfathiazole values represent the average of 3 or more determinations, none of which varied more than one tube from the value given. The peroxide production is expressed in values obtained from 3 or more determinations, none of which varied more than 5 μg/ml from the value given. Ability to produce peroxide was lost by all strains after they became resistant.

Comparison of other biochemical properties. Other biochemical changes were also investigated. Dehydrogenase activity was

---

[1] MacLeod, Colin M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 215.

[2] Main, Edna R., and Shinn, Laurance, E., *J. Biol. Chem.*, 1939, **128**, 417.

TABLE I.
Relation of Peroxide Production to Sulfathiazole Resistance.

Strain		μg/ml H_2O_2 produced		mg/ml sulfathiazole preventing growth	
		Parent	Resistant	Parent	Resistant
1	No growth on 40% bile agar	25	0	.035	>80
2	” ” ” ” ” ”	30	0	.05	>80
6	” ” ” ” ” ”	60	0	.125	>80
12	” ” ” ” ” ”	30	0	10.00	>80
16	Growth on 40% bile agar	15	0	2.50	>80

TABLE II.
Comparison of Relative Dehydrogenase Activity of Parent and Resistant Strains.

Substrate	Strain	Activity	
		Parent	Resistant
Glucose	2	100	231
	6	100	236
Glycerol	2	100	271
	6	100	154
Pyruvate	2	100	533
	6	100	200
Lactate	2	100	205
	6	100	197

Values represent per cent of activity of the parent strain.

determined by the Thunberg technic. The results for 2 strains are given in Table II. Activity for the parent strain was given a value of 100 and that for the resistant strain expressed as a percentage of this activity. The dehydrogenases of the resistant organisms for all 4 of the substrates were always more active than those of the parent strains. The 4 strains which would not grow on 40% bile agar, grew luxuriantly on it after becoming resistant to sulfathiazole. The resistant strains grew much more profusely in tryptose broth. These variations indicate an overall increase in metabolic activity in the resistant organisms which might account for their greater resistance to sulfonamide. Slower growth rates for sulfonamide[3] and penicillin[4] resistant organisms and loss of physiological functions for penicillin[5] resistant strains have been reported. MacLeod's sulfapyridine resistant pneumococcus[1] grew at the same rate as the parent strain and showed no change in the activity of its glucose dehydrogenase. The parent strain had active dehydrogenases for glycerol, lactate, and pyruvate, but no activity for any of these substrates was shown by the resistant strain. Apparently, the resistance to sulfathiazole developed by the streptococci in our investigation involves a different mechanism than that found by any of these workers. It is a relatively stable characteristic, since all strains have maintained their high levels of resistance for more than 8 months while being subcultured on blood agar.

Mechanism of resistance. The reduction of peroxide production associated with increased resistance raises interesting questions concerning the mechanism of acquired resistance. Is this loss of ability to produce hydrogen peroxide related directly to a change in the oxidative enzymes normally used by the organism or is it a loss of function that occurs concomitantly with other changes, the other changes being the important ones for resistance? Davies and Hinshelwood[6] state that if the inhibited enzymes are not essential they will suffer gradual elimination. The enzymes bringing about the production of peroxide may be of this kind, and if so, would not be directly associated with the ability to resist sulfonamides. However, after consideration of the following findings, we feel that resistance is directly related to changes in the respiratory system involving riboflavin.

It seemed possible that the resistant organisms might be producing catalase. This would account for the decreased peroxide production. Since the peroxide is bacteriostatic, destruction by the catalase in the resistant organisms might allow growth at higher concentrations of sulfathiazole. However, catalase determinations were negative, and the addition of 60 μg/ml of hydrogen peroxide to broth did not make the resistant strains more susceptible to sulfathiazole. Hydrogen peroxide is produced by the transfer of hydrogen from a substrate through the flavoprotein enzymes directly to oxygen, without the aid of the cytochrome system. Since the resistant organisms no longer produced hydrogen peroxide, it might be postulated that they had shifted to an energy producing mechanism that did not involve flavoprotein or riboflavin. To test this hypothesis we attempted to grow strains 1, 2, 6 and 12 (all but the enterococcus) in a riboflavin-free broth.* This broth was made for riboflavin assays using *Lactobacillus casei,* and would not allow growth of this organism unless riboflavin was added. None of the 4 organisms grew in the riboflavin-free broth, but usually grew when 20 μg/ml of riboflavin was added. Growth did not take place as readily as in tryptose broth. The resistant strains from these parent organisms grew luxuriantly in the riboflavin-free broth. This indicated that the resistant organisms were either synthesizing their own riboflavin, or were growing without it. Assays of the resistant organisms grown in the riboflavin-free medium showed the presence of riboflavin in the cells and a small amount in the broth. This is similar to McIlwain's findings[7] that organisms capable of synthesizing pantothenic acid are insensitive, while those incapable of synthesizing it are sensitive to pantoyltaurine and that organisms capable of synthesizing a-aminocarboxylic acids were insensitive to the inhibitory action of a-aminosulfonic acids. However, riboflavin in amounts up to 100 μg/ml did not reverse the action of the sulfathiazole when added to broth in which the susceptible organisms had been seeded. This may mean that the sulfathiazole interferes with the utilization of riboflavin rather than with the synthesis which, according to Sevag,[8] is much more difficult to overcome by an antagonist. Cytochromes introduced between the flavoprotein and oxygen in the respiratory system of an organism bring about the production of water instead of hydrogen peroxide. The decreased peroxide production in the resistant organisms might be due to a hitherto unused cytochrome system being put into active use. Heavy suspensions of the resistant organisms would not oxidize p-phenylene diamine. This means that there is not a complete cytochrome c-cytochrome oxidase system present, but does not rule out the possibility of the presence of other cytochromes.[9] Experiments to further explain this mechanism, and to attempt to reverse the biochemical changes observed in the resistant organisms are in progress.

---

[3] Yegian, Diran, Budd, Vera, and Middlebrook, Gardner, *J. Bact.*, 1946, **51**, 479.

[4] Seligmann, Erich, and Wasserman, Michael, *J. Immunol.*, 1947, **57**, 351.

[5] Bellamy, Dexter W., and Klimek, John W., *J. Bact.*, 1948, **55**, 153.

[6] Davies, D. S., and Hinshelwood, C. N., *Trans. Faraday Soc.*, 1947, **43**, 257.

It is recognized that in the absence of single cell isolation there is the possibility that the sulfathiazole is selecting a resistant enterococcus initially present. This would appear most unlikely, however, as no growth was observed with any of the susceptible organisms on 40% bile agar; the trained organisms were repeatedly reisolated by picking from single colonies; and the development of resistance was a gradual one. If an enterococcus were present, it would be likely to grow out as a resistant culture in the first few transfers.

Summary. Peroxide production by 5 strains of greening streptococci was reduced with the acquisition of resistance to sulfathiazole. Other biochemical changes indicated an overall increase in metabolism in the resistant cells. The resistant cells did not acquire the ability to produce catalase, nor a cytochrome c-cytochrome oxidase system, but did acquire the ability to synthesize riboflavin. The possible relation of these changes to respiration is discussed.

---

* Generously supplied by the Difco Laboratories.

[7] McIlwain, H., *Brit. J. Exp. Path.*, 1943, **24**, 212.

[8] Sevag, M. G., *Enzyme Problems in Relation to Chemotherapy. "Adaptation," Mutations, Resistance, and Immunity,* Advances in Enzymology 6, 109, New York, Interscience, 1946.

[9] Sevag, M. G., and Shelburne, M., *J. Gen. Physiol.*, 1942, **26**, 1.

16496

Oral Administration of Bacitracin.

GLENN C. BOND, MILTON J. VANDERBROOK, JEAN L. WILEY, AND MARY ANN NOOK.
(Introduced by John F. Norton.)
From the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

Bacitracin is the antibiotic substance or substances produced by the growth of the "Tracy I" strain of *Bacillus subtilis.* This material is now being produced on a pilot scale and has been made available for study. Several reports have been published regarding the efficacy of this antibiotic, when administered topically or systemically, but information concerning its oral use is very meagre.

The purpose of this paper is to present a report of the findings following oral administration of bacitracin to dogs. The results concern the effect of bacitracin on the intestinal flora, and bacitracin levels in the feces, blood and urine.

Scudi, Clift and Krueger[1] reported, that following the oral administration in dogs of 3,000 u and 6,000 u of bacitracin per kg body weight, no detectable bacitracin was found in the blood stream, nor was any found in the urine during the 24 hours after administration of the antibiotic. Following oral administration of 1500 u/kg body weight to 2 dogs, less than 5% of the oral dose was recovered in the stool. From these findings they concluded that the antibiotic was largely destroyed in the intestinal tract.

Meleney[2] reported the use of oral bacitracin in conjunction with systemic administration, in the treatment of a case of colitis. Clinically, good results were obtained.

Six dogs have been studied. All dogs were put on a uniform diet several days preceding and during the experiment. Bacitracin in solution was administered by stomach tube twice daily, at 8:30 A.M. and 4:30 P.M. The amounts given varied from 2000 u/kg body weight per day to 20,000 u/kg body weight per day. Heparinized blood samples were taken at one and 2 hours after administration of the morning dose. Fecal specimens were obtained by means of a fecal hook each morning before the administration of bacitracin and in 4 of the 6 dogs, 24 hour urine samples were collected. The dogs exhibited no gross change in activity. Body weight was maintained and appetite as measured by food consumption was not affected.

To study the effect of bacitracin on the intestinal flora, a procedure was used similar to that of Poth,[3] for the study of the effect of sulfonamides and streptomycin on the intestinal flora of dogs.

A portion of each specimen of feces was diluted 1-10 with 0.5% potassium sulfite. We have shown that in optimum proportions bacitracin is inactivated by potassium sulfite. After standing for 30 minutes at room temperature, tenfold serial dilutions in saline were carried through 10^{10}, and samples from these dilutions were seeded into test media.

To determine the relative percentages of gram + and gram — organisms, fluid thioglycollate medium was inoculated and incubated 48 hours at 37°C. Smears were made and examined to determine the highest dilution at which gram + and gram — organisms were found.

The presence of fecal streptococci was determined by inoculating tubes of sodium azide medium and incubating at 37°C for 48 hours. Direct smears were made from all tubes showing growth and examined for the presence of streptococci.

Anaerobic agar was inoculated and heated to 80°C for 15 minutes and incubated 37°C for 48 hours to determine the presence of spore forming anaerobes.

---

[1] Scudi, J., Clift, M., and Krueger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 9.

[2] Meleney, F., *Quarterly Progress Report to Office of Surgeon General,* April 1, 1948.

[3] Poth, E., Knotts, F., Lee, J., and Inui, F., *Arch. Surg.,* 1942, **44**, 187.

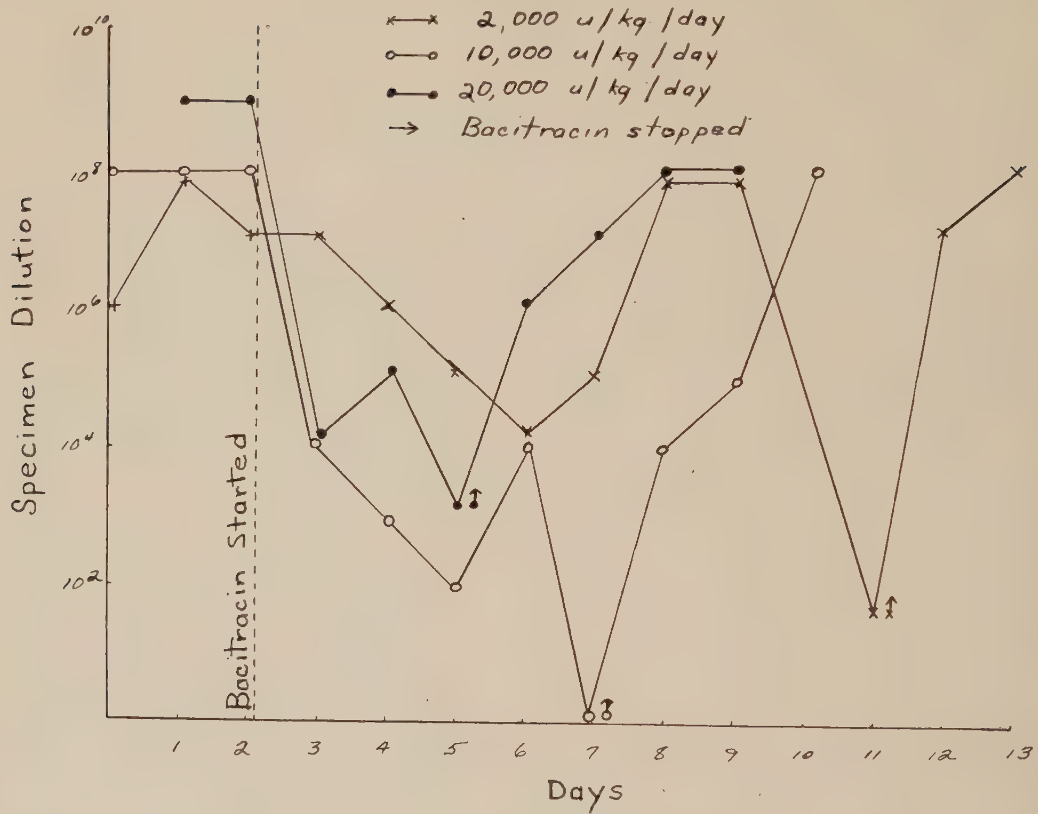


FIG. 1.
Effect of Bacitracin on Fecal Streptococci.

To determine the number of coliform organisms, poured plates of eosin-methylene blue agar were prepared.

Fecal, blood and urine specimens were assayed for bacitracin by the method described by Bond and Nook.[4] A hemolytic streptococcus was the test organism and the standard bacitracin was that supplied by the Food and Drug Administration.

The effect of bacitracin on the intestinal flora of dogs may be seen in the following figures and tables:

Fig. 1 shows the effect of bacitracin on fecal streptococci. At the dosage level of 2,000 u/kg per day, there was a marked variation in total count with the lowest count on the last day of treatment. At a dosage of 10,000 u/kg per day the count dropped abruptly and was zero on the fifth day of treatment. When treatment was stopped there was a rapid rise so that normal levels were reached in 3 days. The 20,000 u treatment curve is similar to the 10,000 u except that treatment was stopped on the third day, which was too soon to allow the count to drop to zero.

Fig. 2 shows the effect of bacitracin on the spore forming anaerobes. The results are comparable to those found for the fecal streptococci. A 2,000 u dose decreased the count but the level did not remain constant during the course of treatment. Both the 10,000 u dose and the 20,000 u dose produced an immediate and rapid drop to zero which remained until cessation of treatment. At that time there was a rapid and sharp rise to normal levels within 2 to 3 days.

Fig. 3 expresses graphically the relative percentage of gram + and gram — organisms with a dose of 10,000 u/kg per day. The pretreatment samples showed a greater incidence

---

[4] Bond, G., and Nook, M., *Science*, 1948, **107**, 228.

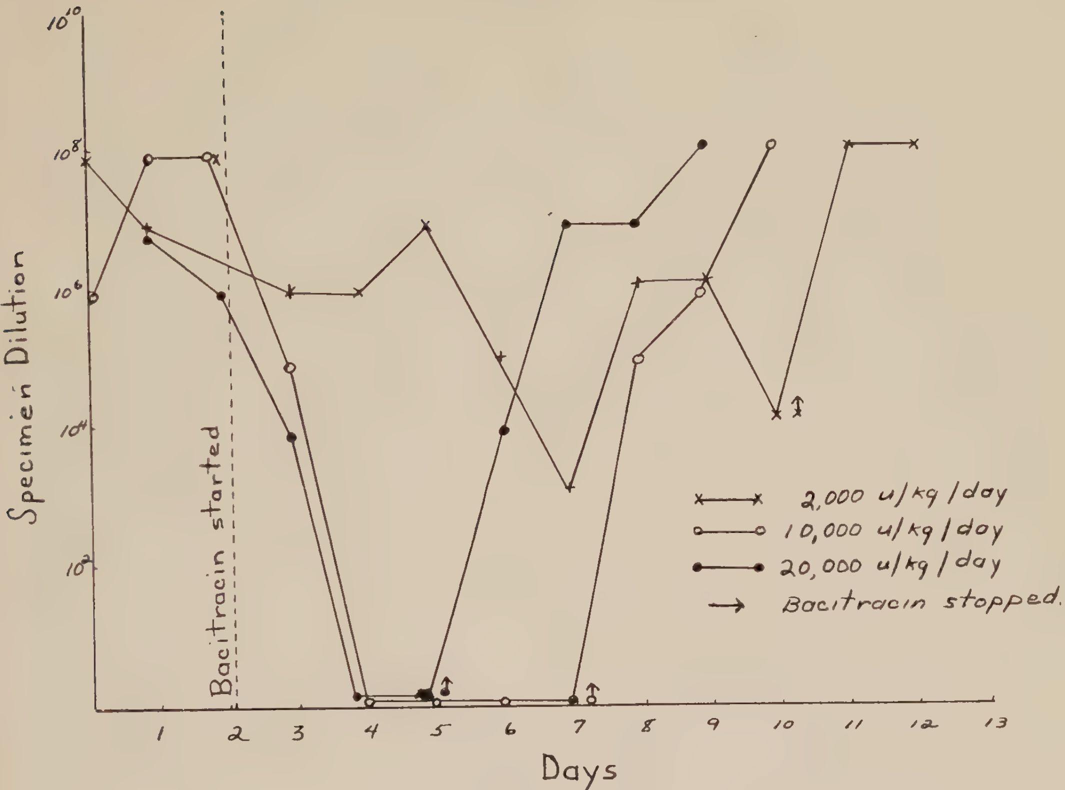


Fig. 2.
Effect of Bacitracin on Spore Forming Anaerobes.

of gram + organisms; but within 24 hours after treatment was started, there was a shift so that gram — organisms predominated during the course of treatment. Forty-eight hours after cessation of treatment the ratio was back to the normal relationship. The figures in the graph are highest dilutions at which the organisms were found.

No significant change was noted in the coliform organisms.

Table I shows the amount of bacitracin per g of feces. At the 2,000 u dose, the feces of 1 dog contained measurable amounts of bacitracin in 3 of 4 days and for 2 days after cessation of treatment. The other dog on the 2,000 u dosage did not show measurable levels until the last day of treatment and the first day after treatment had stopped. With 10,000 u both dogs showed definite levels of bacitracin persisting 1 to 3 days after cessation of treatment. With 20,000 u both dogs showed high levels of bacitracin for the 3 days of treatment and these persisted for 2 days after treatment. It was not possible to determine the total bacitracin in the feces.

Table II shows the blood levels expressed in units of bacitracin per cc of plasma obtained following the oral administration of bacitracin. 2,000 u/kg per day was not sufficient dosage to produce detectable blood levels. 10,000 u/kg per day produced levels of similar magnitude in 2 dogs 1 hour after administration. The 2 hour levels in all cases were lower than the 1 hour level and in one 2 hour sample no bacitracin could be demonstrated. No levels were found 24 hours after cessation of treatment. With 20,000 u/kg per day the plasma levels were higher in all cases than with 10,000 u. Again the 2 hour levels were lower than the 1 hour and no bacitracin could be demonstrated 24 hours after cessation of treatment.

In Table III urine levels of bacitracin are given for 4 of the 6 dogs studied. The urine was collected at 24 hour intervals and assayed.

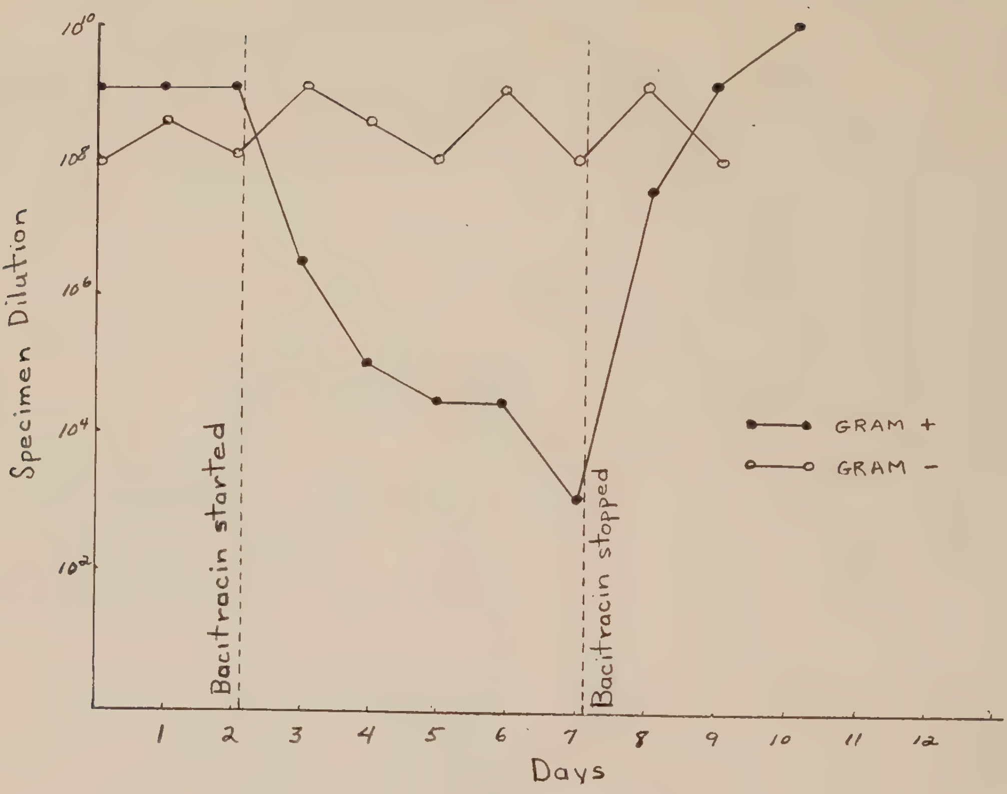


FIG. 3.
Effect of Bacitracin (10,000 u/kg/day) on Gram+ and Gram— organisms.

TABLE I.
Fecal Level of Bacitracin Following Oral Administration.
Expressed as units of Bacitracin per gram of feces.

Dose, u/kg/day	Pre-treatment	Treatment period								Post-treatment		
		Day 1	2	3	4	5	6	7	8	Day 1	2	3
2,000	0	.09	.56	0	.6					1.19	.6	0
2,000	0	0	0	0	0	0	0	0	.1	.4	0	
10,000	0	1.5	1.9	2.5	3.0					3.4	2.7	.6
10,000	0	1.0	12.0	4.2	8.7					.4	0	
20,000	0	75.0	75.0	15.0						7.0	8.0	0
20,000	0	90.0	85.0	100.0						18.0	9.0	0

2,000 u daily gave very low and irregular results. With 10,000 u a urinary level was found for each day of treatment and for 3 days following treatment. 0.22 u/cc of urine was the highest level obtained at this dosage. The 2 dogs on the 20,000 u dose showed high urinary levels for the 3 days of treatment and in one case detectable levels persisted for 2 days post treatment. The highest level obtained was 1.65 u/cc of urine.

Summary. From the small series of animals receiving oral bacitracin, the following significant facts were obtained: 1. Fecal streptococci and spore forming anaerobes were greatly reduced in the intestine at a dosage between 2,000 and 10,000 u/kg per day. 2. Coliform organisms were little affected by bacitracin. 3. Bacitracin persisted in the feces as long as 3 days following cessation of treatment. 4. Bacitracin was absorbed from

TABLE II.

Blood Levels of Bacitracin Following Oral Administration. Expressed as units per cc of plasma.

Dose, u/kg/day	Pre-treatment	Treatment period								Post-treatment	
		Day 1		Day 2		Day 3		Day 4			
		1 hr	2 hr	1 hr	2 hr	1 hr	2 hr	1 hr	2 hr	Day 1	Day 2
2.000 u.	0	0	0	0	0	0	0	0	0	0	0
2,000 u.	0	0		0		0		0		0	0
10,000 u.	0	0		.013		.048		.045		0	0
10,000 u.	0	.085	.08	.064	.045	.046	0	.058	.047	0	0
20,000 u.	0	.1	.095	.088	.07	.22	.1			0	0
20,000 u.	0	.11	.098	.12	.08	.55	.17			0	0

TABLE III.

Urine Levels of Bacitracin Following Oral Administration.

Dose, u/kg/day	Pre-treatment	Treatment period								Post-treatment					
		Day 1		Day 2		Day 3		Day 4		Day 1		Day 2		Day 3	
		u/cc	Total u	u/cc	Total u	u/cc	Total u	u/cc	Total u	u/cc	Total u	u/cc	Total u	u/cc	Total u
2,000	0	0	0	.045	10.5	0	0	0	0	.046	11.9	0	0	0	0
10,000	0	.06	8.21	.22	49.5	.096		.03	6.75	.1	9.0	.04	12.7	.025	4.75
20,000	0	.55	2550	.8	2080	.18	15.0			.075	15.0	.054	17.8	0	0
20,000	0	.9	3690	.5	1550	1.65	792			.17	73	0	0	0	0

the intestinal tract as evidenced by significant blood levels. 10,000 u/kg per day was sufficient to produce blood levels as high as 0.085 u/cc of plasma 1 hour after administration. 5. Additional evidence of absorption was obtained by the finding of high levels of bacitracin in the urine. The highest obtained with 10,000 u was 0.22 u/cc and with 20,000 u 1.65 u/cc of urine.

16497 P

Attenuation of Vaccinial Virus by Interfacial Adsorption.*

J. M. JOHLIN.

From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tenn.

Previous mention has been made of the possibility of attenuating the virus of vaccinia by interfacial adsorption.[1] The data obtained at that time are those given in the present report. They illustrate the attenuation of this virus so that it will no longer produce the lesions which are characteristic of untreated vaccinia when injected endermically into rabbits but will nevertheless produce an immunity to the action of the untreated virus in these animals.

The method of attenuation employed was the same as that which was used in inducing the clotting of non-clotting blood plasmas[2] and in the attenuation of insulin[3,4] and of toxins such as ricin,[1] tetanal toxin,[1] staphylococcal toxin,[5,6] and snake venom.[1] It was shown there that, while emulsified chloroform is on the whole to be preferred over other adsorbing agents, inert emulsified gases are in some instances equally efficient in bringing about an attenuation.[6]

The virus† used in the present experiments was produced in chick embryos and suspended in saline. The saline suspension was treated by shaking with an equal volume of chloroform for 24 hours and all chloroform subsequently removed by evaporation under reduced pressure at a temperature below 40°C. Newly acquired animals which had not been exposed to vaccinia were used and each animal was isolated from all others during the period of treatment.

After completing these experiments, the writer found that Kelser[7-10] had prepared a rinderpest vaccine by allowing a saline suspension of the finely ground tissue from animals killed in the acute stages of rinderpest to stand in the ice box for a few hours in the presence of .75% chloroform. Kelser later prepared a rabic vaccine by the similar treatment of a 33⅓% fixed-virus brain-emulsion in physiological saline in the presence of 1.0% chloroform.

The writer subsequently found that Kelser's method could be applied to vaccinia to attenuate it sufficiently so that it will produce no lesions when administered endermically. Long standing of toxins with chloroform had,

---

* Aided by a grant to the Vanderbilt University School of Medicine from the Division of Medical Sciences of the Rockefeller Foundation.

[1] Johlin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 569.

[2] Johlin, J. M., *J. Biol. Chem.*, 1929, **81**, 99.

[3] Johlin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 524.

[4] Johlin, J. M., *Endocrinology*, 1941, **29**, 574.

[5] Johlin, J. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 135.

[6] Johlin, J. M., and Rigdon, R. H., *J. Immunol.*, 1941, **41**, 233.

† The writer is indebted to Dr. John Buddingh for the virus preparations used in these experiments.

[7] Kelser, R. A., *Philippine J. Sci.*, 1928, **36**, 373.

[8] Kelser, R. A., Youngberg, S., and Topacio, T., *J. Am. Vet. Med. Assn.*, 1929, **74**, 28.

[9] Kelser, R. A., *J. Am. Vet. Med. Assn.*, 1930, **77**, 595.

[10] Kelser, R. A., *Mil. Surgeon*, 1931, **69**, 34.

on the other hand, not been found to have any appreciable effect in attenuating these substances. The effect of Kelser's treatment would seem to be also that of interfacial adsorption.

In the present experiments each of a series of 3 rabbits was given 6 endermical injections widely distributed, totalling 1.5 cc in each case, of the treated viral suspension. The injections were repeated at 4-day intervals, 2 rabbits receiving 3 additional series of injections and the third receiving but a second series. No symptoms followed the first 2 series of injections in any animal. In the case of the 2 animals receiving a third and fourth series of injections, an area of slight induration, about 15 mm in diameter, without erythema, developed within 24 hours at each site of injection and lasted about 2 days. After a rest period of 7 days following the last injection, each animal received 2 injections of 0.1 cc each of the untreated viral suspension. Of the 2 animals which had received the longer series of injections of the treated virus, one developed areas of edema, 30 × 50 mm and 30 × 40 mm accompanied by a faint erythema, at the sites of injection of the untreated virus, within 24 hours. The smaller of these areas disappeared after 4 days and the other after 7 days. The other animal developed areas of slight induration, 18 × 20 mm and 15 × 20 mm accompanied by a faint erythema, at the sites of injection of the untreated virus, within 24 hours. These reactions disappeared within 3 days after the injections and, like those which developed after the third and fourth series of injections of the treated virus, may have been due to an acquired protein-sensitivity.

The third rabbit, which had received but 2 series of injections of the treated virus, developed areas of slight induration, 15 × 30 mm and 30 × 40 mm, accompanied by a faint erythema, at the sites of injection of the untreated virus, within 24 hours. These reactions disappeared within 3 days after the injection of the untreated virus. None of these animals developed lesions. The average increase in weight of the 3 rabbits during this course of treatment was 55 g.

A control animal received 2 injections of 0.1 cc each of the untreated virus and at the same time received 2 similar injections of the treated virus. The treated virus produced no symptoms while the untreated virus produced its usual symptoms. Ten days later this rabbit again received 2 similar injections of the untreated virus. Areas of slight induration and faint erythema developed within 36 hours but disappeared within 3 days after the injections.

Summary. Vaccinia can be attenuated by interfacial adsorption so that it will no longer produce the lesions of this virus when injected endermically into rabbits but will produce an immunity to the action of the untreated virus.

16498

Effect of Medium on Resistance of *Staphylococcus aureus* to Quaternary Ammonium Compounds.*

W. L. Mallmann, Anita Leavitt, and Dwight A. Joslyn.

From the Department of Bacteriology and Public Health, Michigan State College, and the Research Department, Parke-Davis and Company, Detroit.

A comparison of data obtained by two of the authors on a quaternary ammonium compound with *Staphylococcus aureus* (No. 209 F.D.A.) consistently revealed strikingly different results. It was found that the culture used by one author was grown in standard F.D.A. broth while the other author used Difco "disinfectant testing" broth. A study of the 2 media was made to determine the cause of the discrepancy.

---

* Journal Article No. 952 (N.S.) from Michigan Agricultural Experiment Station.

Tests were made simultaneously on both media using *Staph. aureus* (No. 209 F.D.A.) with a mixture of alkyl dimethyl benzyl ammonium chlorides.

The culture was transferred daily in each medium for at least 3 successive days to obtain a constant inoculum. The tests were made according to the phenol coefficient procedure recommended by the Food and Drug Administration.[1] The culture grown in Difco "disinfectant testing" broth was adjusted to pH 7.2 before use to eliminate the effect of differences in hydrogen ion concentration. Killing was obtained in 10 minutes in Difco "disinfectant testing" broth in a dilution of 1-4000, whereas in F.D.A. broth the killing dilution was 1-20,000. The same effect was obtained with N(acyl colamine formyl methyl) pyridium chloride. With Difco "disinfectant testing" broth the killing dilution was 1-250, whereas with F.D.A. broth the dilution was 1-1800. No differences in killing dilutions were obtained with phenol. It is apparent from the results obtained that the effect is due to the action of the medium on the resistance of the bacteria.

To further demonstrate that the action is due to a change in the resistance of the test organism, the organism grown in each broth was centrifuged and washed repeatedly to remove the broth. The organisms were resuspended in F.D.A. broth and tested as before. The same results were obtained, namely a high resistance in those organisms grown in Difco broth and a loss resistance in those grown in F.D.A. broth.

In the next series of tests, variations were made in the formulations of Difco "disinfectant testing" broth to determine the specific ingredient responsible for the change in the resistance of the organisms. The formula for Difco "disinfectant testing" broth follows: proteose-peptone Difco 10 g, Bacto beef extract 3 g, Bacto lactose 5 g, sodium chloride 2 g, ascorbic acid 0.25 g, and water 1,000 ml.

Substitutions were made in the kinds of peptone used and formulations were made with and without lactose. The peptones used were Armour's peptone, Bacto proteose peptone No. 3, and Bacto peptone. Two media were prepared using Armour's peptone and proteose peptone respectively but the lactose was not added. The tests were made with 4(1, 1, 3, 3-tetra methyl)butyl phenoxyethoxy ethyl dimethyl benzyl ammonium chloride. It was found that the kind of peptone does not affect the resistance of the organisms. On the other hand, the addition of lactose causes the change in resistance of the organism because media containing lactose gave killing dilutions of 1-1000 to 1-3000, whereas in the absence of lactose, the killing dilutions were 1-20,000 or higher. When other fermentable sugars (glucose and maltose) were substituted for lactose, the results were similar to those obtained with lactose. When lactose was added to the F.D.A. broth, an increase in resistance of the organisms occurred to the same extent as that obtained with Difco "disinfectant testing" broth.

Other gram positive cocci that ferment lactose were tested. *Micrococcus caseolyticus* gave results comparable to those obtained with *Staph. aureus*.

In another series of tests, *Eberthella typhosa,* a gram negative organism, was tested. Because lactose is not fermented by this organism, glucose was substituted. No difference was obtained with the various media. It is apparent that the presence of a fermentable sugar does not change the resistance of *Eberth. typhosa* toward quaternary ammonium compounds. Tests with *Escherichia coli,* a gram negative organism fermenting lactose, gave results similar to those obtained with *Eberth. typhosa.* These observations indicated that the resistance of gram negative organisms is not changed by growing in Difco "disinfectant testing" broth.

Tests were made to determine whether the culture medium used for growing *Staph. aureus* had any effect on other types of disinfectants. Two compounds, Lysol, a cresolic type, and Benchlophen, a phenolic derivative, were studied. No difference was obtained in killing dilutions with these two disinfectants in the F.D.A. broth and Difco "disinfectant testing" broth. It would appear that the change in resistance is limited largely to the quaternary ammonium compounds or possibly to those disinfectants characterized by long hydrocarbon chains. Further studies along this line are in progress.

Conclusions. Data have been presented to show that the resistance of *Staph. aureus* to quaternary ammonium compounds is materially increased by growing the test cultures in Difco "disinfectant testing" broth as compared with standard F.D.A. broth.

---

[1] Ruehle, G. L. A., and Brewer, C. A., *U. S. Dept. Agri. Circ.* 198, 1931.

16499

Gastrointestinal Tract of Guinea Pig and Elimination of Ascorbic Acid Given Intraperitoneally.

Mary Elizabeth Reid. (Introduced by F. S. Daft.)

From the National Institute of Health, U. S. Public Health Service, Bethesda, Md.

In previous experiments it was found that adult guinea pigs receiving their ascorbic acid intraperitoneally in the proportion of 5 mg per 100 g of body weight excrete about one-fourth of it in the urine. Although some of the vitamin may be used up metabolically in some way in the tissues, it seemed probable that considerable loss due to destruction was occurring elsewhere. The present tests were performed to determine where this destruction, if any, takes place. A preliminary report of the results was made previously.[1]

Method. The investigations were conducted by administering the ascorbic acid intraperitoneally and determining at successive intervals the rate of urinary excretion of the vitamin, the amount present in the blood, liver, kidneys, spleen, and adrenals, and amount present in the contents of 4 different parts of the gastrointestinal tract, then correlating the latter data with observations made in another study[2] on the rate of movement of the contents through the gastrointestinal tract. In these latter studies it was found that in the guinea pig there is a rapid movement of the contents through the stomach and small intestine. Within a period of 2 hours these organs are practically emptied.

The present observations were made on 41 adult guinea pigs of an inbred strain. They were fed a commercial pelleted diet devoid of ascorbic acid. To ensure a sufficient supply of the B vitamins, 1% of powdered yeast was added to the pellets. The 29 male animals used in the tests had an average weight of 921 g, the 12 females an average weight of 771 g. Each animal was injected intraperitoneally each day with ascorbic acid (made up in 0.5% $NaHCO_3$, 15 mg asc. ac./ml) in the amount of 5 mg per 100 g body weight. The animals were maintained on this regime for at least a month before being used in the tests. At periods varying between 1 to 24 hours after injection, blood samples were taken from the heart, following which the animals were killed and the organs quickly removed. The liver, kidneys, spleen, and adrenals were frozen rapidly in dry ice and kept in the frozen state until the extractions with 10% metaphosphoric acid could be made. The contents of the stomach, small intestines, cecum, and large intestine were washed out separately with 10% metaphosphoric acid and centrifuged. The centrifugate was washed with another volume of acid and centrifuged. The first and second extracts were combined and made up to appropriate volumes. In the later phases of the study the washing of the centrifugate was omitted, the contents being made up to appropriate volume

---

[1] Reid, M. E., *Proc. Fed. Am. Soc. Exp. Biol.*, 1947, **6**, 418.

[2] Reid, M. E., and White, W. C., *Am. J. Physiol.*, in press.

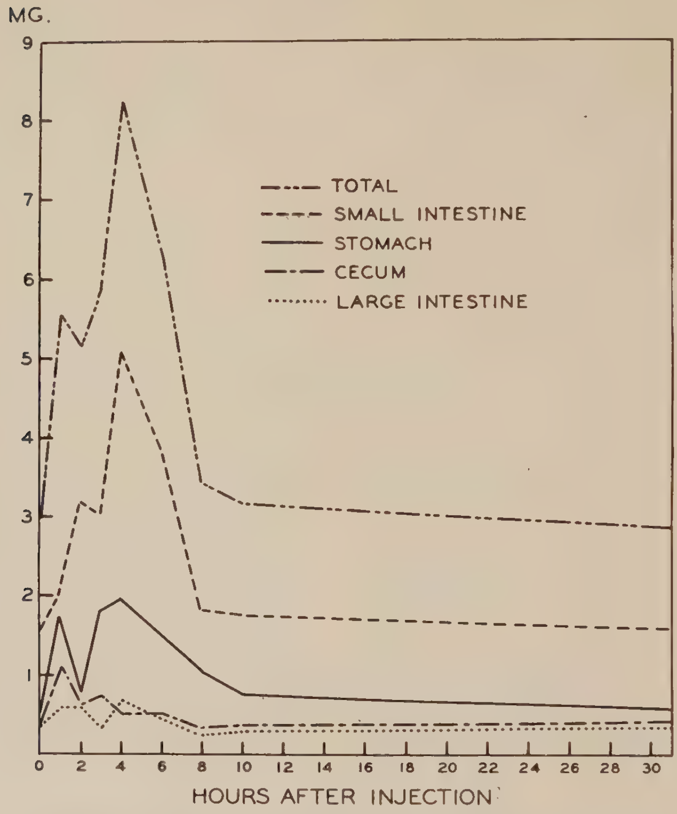


FIG. 1.

Total ascorbic acid (osazone method) found in contents of gastrointestinal tract at successive periods after intraperitoneal injection of the vitamin.

with the metaphosphoric acid before centrifuging with the results not significantly different from those obtained with the previous procedure. Assays were made by the indophenol method on these extracts and also on those of the organs. The extracts remaining after the indophenol tests, also the supernatant fluid from the whole blood samples prepared according to the Roe-Kuether technic[3] were frozen and kept in this state until the following day. Acetic acid was then added to make a final concentration of 10% acetic and 5% metaphosphoric acid and assays were made by the osazone method.

The number of animals studied in each group ranged from 3 to 8.

Results. Measurable osazone values were found in the contents of the gastrointestinal tract in all of the tests. Fig. 1 shows the average result (expressed as mg ascorbic acid/kg body weight) obtained with the osazone method. From a relatively small amount present immediately before injection, the ascorbic acid in the contents of the alimentary tract increased up to and through the fourth hour after injection and thereafter decreased slowly. After 10 hours, the amount had decreased to a level not much above the starting level. Most of the fluctuation with time is found in the contents of the stomach and small intestine. From 70 to 85% of the total amount of ascorbic acid in the contents of the digestive tract is found in the stomach and small intestine and more in the intestine than in the stomach. The indophenol values for the contents of the stomach and small intestine were usually lower than the osazone values, presumably because of the presence of some of the vitamin in the dehydro form. On the other hand, the indophenol values for the contents of the cecum and large intestine were always much higher than the osazone values, doubtless because of the presence of reducing substances other than ascorbic acid. The osazone method may give values somewhat higher than the true ascorbic acid because of the presence of small amounts of osazone-forming substances other than ascorbic acid such as uronic acids and diketogulonic acid. Since diketogulonic acid is known to be an oxidation product of ascorbic acid and since it forms an osazone, the osazone values obtained particularly in the contents of the cecum (where the digestion of cellulose with presumable liberation of uronic acids occurs) and large intestine may not be due entirely to ascorbic acid. The high values found in the contents of the small intestine and the low values in the much greater volume of contents of the cecum indicates that destruction of the vitamin must occur rather rapidly as the contents pass into the cecum.

---

[3] Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, **147**, 399.

Table I shows the ascorbic acid content of the blood and some of the organs at successive periods after injection of the vitamin as determined by the osazone method. The average value in whole blood 24 hours after injection is 0.83 mg %. Within 1 hour after injection it increases more than 5-fold then falls about 40% by the end of the third hour and remains at approximately this level through the sixth hour. By the tenth hour it drops to 1.31 mg % and thereafter decreases until it reaches the 0.83 starting level after 24 hours. The liver and kidneys both show marked increases in ascorbic acid content after injection. The kidneys gained 130% within an hour, lost some of it rather rapidly during the second hour and thereafter decreased slowly. The highest value for the liver was found at the end of the second hour. At the end of the 24-hour period the value was only half that found 1 hour after injection of the vitamin. The spleen showed only slight fluctuations with time. There were irregularities in the content of the adrenals but the variations do not appear to bear a close relation to the time

TABLE I.

Ascorbic Acid in Blood and Organs (Osazone Method) at Successive Periods After Intraperitoneal Injection of the Vitamin.

No. of animals	Hr after last inj.	Blood		Liver		Kidneys		Spleen		Adrenals	
		Mg/100 ml	s.d.	Mg%	s.d.	Mg%	s.d.	Mg%	s.d.	Mg%	s.d.
4	0 (24)*	0.83	.10	30.7	5.4	11.8	2.1	59.9	1.2	145.4	9.0
4	1	4.29	.34	48.4	2.3	27.2	2.1	65.3	4.7	166.5	19.0
4	2	3.54	.18	58.5	4.6	20.6	1.0	60.5	3.4	144.8	8.5
4	3	2.29	.28	50.3	2.6	20.5	1.1	70.8	5.6	167.7	11.5
4	4	2.44	.29	51.7	3.4	18.0	2.2	68.4	4.3	146.6	14.7
4	6	2.11	.15	49.4	1.8	16.7	1.3	62.8	3.3	144.1	4.2
3	8	1.48	.16	51.4	4.0	15.3	1.4	66.6	5.9	141.0	4.3
3	10	1.31	.17	39.0	5.9	12.1	0.2	62.5	5.0	152.6	11.4
4	24	0.83	.10	30.7	5.4	11.8	2.1	59.9	1.2	145.4	9.0

* This group is identical to the 24-hour group reported below. It is included in this place for ease of comparison.

TABLE II.
Average Rate of Urinary Excretion of Ascorbic Acid.

Hr after inj.	% of total amt excreted
0- 1	4.4
1- 2	14.6
2- 3	27.2
3- 4	20.0
4- 6	28.9
6- 8	2.9
8-10	0.9
10-24	1.1
Total for 24 hr	100.0

of administration of the vitamin. The ascorbic acid values for all these organs are considerably higher than have been found by other investigators using the oral route of administration. The value for whole blood after 24 hours, however, is only slightly higher than that reported by Kuether, Telford, and Roe[4] for the blood of guinea pigs maintained on a diet of cabbage.

Table II summarizes the results of a study of the urinary excretion of ascorbic acid made daily for 6 successive days in each of 10 animals maintained under the experimental conditions previously described. Each of the values given in the table thus represents an average of 60 determinations using the indophenol method. In confirmation of previous results, approximately one-fourth of the total dose administered was found in the urine. The total excretion for the 24-hour period is expressed as 100%. The most active period of excretion extends from the second through the sixth hour. This is also the period of high blood level.

Discussion. When large doses of vitamin C are given intraperitoneally, a marked increase in the levels in the blood and certain tissues with considerable urinary excretion occurs. This contrasts to the situation when large doses are given orally in which case the blood and tissue levels remain much more nearly normal and there is no urinary excretion. It has been assumed by different investigators that the excess of orally administered vitamin C is not absorbed, but is destroyed in the digestive tract. Evidence to support this contention was obtained in the present studies in which the vitamin is given intraperitoneally. Considerable amounts appear in the contents of the stomach and small intestine, but very much less in the cecum and large intestine in spite of the fact that the rate of movement is such that the vitamin must be passed to these organs. A probable interpretation of these data appears to be that destruction does occur in the lower portion of the gastrointestinal tract as there is no definite evidence of reabsorption as judged by the blood studies.

Since as much as 15% of the injected dose may be found at one time (4 hours) in the stomach and small intestine and since the contents of these organs are being constantly and rapidly passed into the cecum and large intestine, it appears likely that the total amount destroyed by the gastrointestinal tract must be considerably more than 15%. This rate of destruction would be ample to account for the loss of vitamin C when given orally.

Summary. Ascorbic acid was found in the stomach and small intestine of adult guinea pigs maintained on a diet devoid of the vitamin. The amount present varied with the length of time after intraperitoneal injection of the substance, the maximum occurring at 4 hours. Small amounts only were found in the contents of the cecum and large intestine. The results suggest that the vitamin is excreted into the stomach and small intestine and is destroyed in the cecum.

The maximum content of ascorbic acid was found in the blood and kidneys 1 hour after injection, in the liver 2 hours after injection, and the maximum excretion in the urine occurred between the second and third hours after injection.

---

[4] Kuether, C. A., Telford, I. R., and Roe, J. H., *J. Nutrition,* 1944, **28**, 347.

16500

Tuberculin Reaction. II. Cytotoxicity of Tuberculin for Splenic Explants of Desensitized Tuberculous Guinea Pigs.*

WALDEMAR F. KIRCHHEIMER AND RUSSELL S. WEISER.
(Introduced by Erling J. Ordal.)
From the Department of Microbiology, University of Washington School of Medicine, Seattle, Wash.

Important differences between tuberculin sensitivity and other types of sensitivity have been reviewed in a previous publication.[1] One of the differences is that tuberculin exerts a specific cytotoxic action on tissue explants of tuberculin-sensitive animals, whereas, such specific cytotoxic effects of antigen are not present in other types of sensitivity. This "*in vitro* sensitivity" is not limited to tuberculin-sensitive tissues but apparently includes all sensitivities of the tuberculin type. For example, Moen[2] has noted that *in vitro* cytotoxic action can be produced in case of the tuberculin type sensitivity which develops in guinea pigs as a consequence of infection with β hemolytic streptococci. Extracts of the causative organism were found to exert a specific cytotoxic action on tissue explants of infected guinea pigs.

Heilman and Seibert[3] in studies on the *in vitro* action of purified fractions of tubercle bacilli on tuberculin-sensitive tissues demonstrated that, in common with the cutaneous and systemic tuberculin reactions, specific *in vitro* cytotoxic action can be produced with tuberculoprotein, but not by other components such as polysaccharide and nucleic acid fractions.

Lasfargues, Bouquet and Delaunay[4] express doubt that the *in vitro* cytotoxic action of tuberculin is specific. They believe that the toxicity observed is simply the expression of a non-specific heightened vulnerability of the cells of the tuberculous animal. However, this is not consistent with the observation of Heilman, Feldman and Mann[5] that the tissues of animals sensitized with heat-killed tubercle bacilli react in a similar manner, for, it is not probable that the tissues of such non-infected healthy animals would possess a non-specific heightened vulnerability. It seems logical that the specific *in vitro* cytotoxic action of tuberculin is as much a manifestation of tuberculin sensitivity as are the cutaneous and systemic tuberculin reactions and, moreover, that the systemic tuberculin reaction is the summation of an "*in vivo* cytotoxic action" of tuberculin on sensitive cells analogous to the cytotoxic action which occurs *in vitro*.

In a previous publication[1] we have reviewed the evidence indicating that tuberculin type sensitivity is dependent upon antibody. The object of the present investigation was to provide additional evidence on the existence and nature of tuberculin antibody.

It was our opinion that if the *in vitro* cytotoxic action of tuberculin is dependent upon antibody the combination of such antibody with antigen by desensitization treatment with tuberculin should render the tissues of the animal less susceptible to the *in vitro* cytotoxic effect of tuberculin than the tissues of sensitive controls. Furthermore, it seemed probable that the *in vitro* studies proposed would greatly reduce the possible interference of the humoral blocking antibodies (antituberculins, anticutins) which have been assumed by some investigators[6-8] to be responsible for the high

---

* This work was supported by a grant from the Alice McDermott Research Foundation of the University of Washington.

[1] Kirchheimer, W. F., and Weiser, R. S., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 166.

[2] Moen, J. K., *J. Exp. Med.*, 1936, **64**, 355.

[3] Heilman, D. H., and Seibert, F. B., *Am. Rev. Tuberc.*, 1946, **53**, 71.

[4] Lasfargues, E., Boquet, P., and Delauney, A., *Ann. de l'Inst. Pasteur*, 1947, **73**, 169.

[5] Heilman, D. H., Feldman, W. H., and Mann, F. C., *Am. Rev. Tuberc.*, 1945, **52**, 65.

[6] Pickert, M., and Löwenstein, E., *Dtsch. Med. Wochenschr.*, 1908, **34**, 2262.

tolerance to tuberculin observed in some animal species and in tuberculous humans following tuberculin treatment.

Methods. Adult male and female guinea pigs averaging approximately 600 to 800 g in weight were infected with the B.C.G. strain of *Mycobacterium tuberculosis* variety *bovis.* Approximately 5 to 10 mg of a young culture were given either subcutaneously or intraperitoneally. Within 4 to 5 weeks after infection most of the animals responded with a necrotic cutaneous tuberculin reaction to Old Tuberculin 1:100.† The animals were tuberculin-tested from time to time and only those that gave necrotic reactions to O.T. 1:100 were used in the experiments.

Desensitization was accomplished with progressively increasing intraperitoneal doses of O.T. given over a period of 3-4 days. The initial dose was 0.5 cc of O.T. 1:10,000. Desensitization treatment was continued until the animals failed to react to O.T. 1:10 after which they were given a final desensitizing dose of 1 to 2 cc of O.T. 1:2. The animals were used for tissue culture the day following the final desensitizing dose of tuberculin.

The methods of tissue culture used were essentially the same as those employed by Rich and Lewis.[9] Splenic tissue was scissored in cold Locke's solution and matched pieces approximately 1 mm in diameter were transferred to a second bath of cold Locke's solution containing the concentrations of O.T. or glycerine broth present in the plasma clots used in the respective experiments. The explants were finally transferred to large coverglasses with a capillary pipette and a few drops of fresh, cold heparinized guinea pig plasma containing the respective concentration of tuberculin or glycerine broth was added. The final concentration of O.T. and of glycerine broth in the plasma clots ranged from 1:40 to 1:80. A drop of molten paraffin-vaseline mixture was then deposited at each corner of the coverglasses and embryo culture slides inverted over the preparations. The slides were righted and the edges of the coverglasses sealed with molten paraffin. The cultures were incubated as hanging drops at 37.5°C ± 0.2°C in a specially constructed electric incubator. After 24 hours of incubation the mean radius of the zone of cell migration of each explant was calculated from measurements of the radius made at several points with an ocular micrometer using a magnification of X32.

Two animals were employed in each experiment; one sensitive and one desensitized. Several explants from the spleen of each animal were made in tuberculin and in glycerine broth. It was necessary to include glycerine broth control explants for each animal because of the great variation in potential cell migration in explants of different animals. Both animals were killed at the same time and explants from the sensitive and from the desensitized animal were made alternately so as to insure uniform treatment. The time which elapsed between the killing of the animals and the beginning of the incubation of the explants was approximately 1 hour. The averages of the mean radii of the zones of cell migration in the explants containing tuberculin and in the controls containing glycerine broth were determined. The extent of the cytotoxic action of tuberculin on the explants from each animal was determined by calculating the percent alteration in cell migration using the values of the explants in glycerine broth as 100%.

Results. The results of the principal experiments are presented in Table I. They show that the cytotoxic effect of tuberculin, although not absent, is uniformly and significantly less in the explants from desensitized animals than in those from sensitive animals. Additional experiments, not included in the table because of minor variations in technic, all showed a similar result.

In order to determine whether the results with explants from the desensitized animals were due to specific desensitization 2 control

---

[7] Jadassohn, W., and Martenstein, H., *Klin. Wochenschr.*, 1919, **26**, 1210.

[8] Pinner, M., *Am. Rev. Tuberc.*, 1931, **23**, 175.

† We are indebted to the Cutter Laboratories of Berkeley, Calif., who kindly supplied us with the Old Tuberculin used in these experiments.

[9] Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

TABLE I.
Comparison of Effect of Tuberculin on Migration of Cells in Splenic Explants from Sensitive and Desensitized guinea pigs infected with the B.C.G. strain of *M. tuberculosis.*

Exp. No.	No. of explants				Conc. of O.T. or gl.	Avg of the mean radii of the zones of cell migration in mm				% alteration of cell migration	
	d + O.T.	s+O.T.	d+gl.	s+gl.		d+O.T.	s+O.T.	d+gl.	s+gl.	d+O.T.	s+O.T.
I.	4	4	2	2	1:80	1.20	0.37	1.38	0.80	—14	—54
II.	4	3	2	2	1:50	0.85	1.02	0.92	1.84	— 8	—45
III.	4	4	4	4	1:40	0.36	0.23	0.51	0.50	—30	—52
IV.	4	4	4	4	1:50	0.86	0.73	0.78	1.18	+10	—39
V.	4	4	4	4	1:50	0.89	0.48	1.01	0.83	—12	—43

The meanings of the abbreviations used in the various tables are as follows: d = specifically desensitized; "d" = non-specifically desensitized; gl = glycerin broth; s = sensitive; O.T. = Old Tuberculin.

TABLE II.
Effect of Tuberculin on Migration of Cells in Splenic Explants from Guinea Pigs Infected with the B.C.G. Strain of *M. tuberculosis* and Given Desensitization Treatment with "Smega" Tuberculin.

Exp. No.	No. of explants		Conc.of O.T. or gl.	Avg of the mean radii of the zones of cell migration in mm		% alteration of cell migration
	"d"+O.T.	"d"+gl.		"d"+O.T.	"d"+gl.	
I.	10	6	1:50	0.60	1.26	—53
II.	4	4	1:50	0.49	1.20	—58

See Table I for definitions of abbreviations.

TABLE III.
Comparison of Effect of Tuberculin on Migration of Cells in Splenic Explants from Sensitized and Desensitized Guinea Pigs Infected with *M. tuberculosis* H37 Rv.

Exp. No.	No. of explants				Conc. of O.T. or gl.	Avg of the mean radii of the zones of cell migration in mm				% alteration of cell migration	
	d + O.T.	s+O.T.	d+gl.	s+gl.		d+O.T.	s+O.T.	d+gl.	s+gl.	d+O.T.	s+O.T.
I.	4	4	4	4	1:50	0.46	0.23	0.53	0.46	—14	—50
II.	4	4	4	3	1:50	0.57	0.46	0.81	0.75	—29	—39
III.	6	4	4	4	1:50	0.16	0.23	0.35	0.92	—55	—75

See Table I for definitions of abbreviations.

experiments were conducted with explants from animals given desensitization treatment with tuberculin prepared from *Mycobacterium smegmatis*. The results of these experiments are presented in Table II. They show that the depression of cell migration by O.T. in explants from these animals was of the same order of magnitude as the depression shown by explants of untreated sensitive animals thus indicating that the results with the tissues of animals desensitized with O.T. are due to specific desensitization.

Several experiments were also conducted in which explants from guinea pigs infected with *Mycobacterium tuberculosis* H37 Rv were employed. Considerable difficulty was experienced in securing uniformity in the explants because of the variable and often extensive splenic lesions in the animals. In addition the rapid desensitization treatment employed resulted in death of many of the animals. The results of 3 of these experiments are shown in Table III. They are similar to the results obtained with B.C.G. sensitized animals as shown in Table I.

It is to be noted that the tissue culture method employed in the various experiments only permitted observations on the initial migration of cells from the explants. The cellular elements participating in this migration were polymorphonuclear leucocytes, lymphocytes and to a lesser degree monocytes. However, the inactivity of monocytes is not significant since the high concentration of tuberculin employed exerted a marked toxicity on the monocytes of all preparations including explants from normal animals. This confirms the observations of Shwartzman.[10]

Discussion. It is a reasonable assumption that if the *in vitro* cytotoxic effect of tuberculin on the tissues of sensitive animals is the outcome of the interaction of a sessile antibody with specific antigen, specific desensitization should accomplish saturation of antibody and consequent abolition of this cytotoxic sensitivity.

The fact that the desensitization treatment used in the present experiments did not completely abolish *in vitro* cytotoxic sensitivity does not necessarily militate against the theory that this sensitivity is dependent upon antibody. One possibility is that desensitization of the animals was not complete. It would be of interest to determine whether more complete desensitization of the animals can be accomplished and also whether desensitization of tissues could be accomplished *in vitro* by exposure to a series of increasing concentrations of tuberculin.

It does not seem likely that a blocking antibody was responsible for the results obtained in the present experiments in view of the speed with which a refractory state developed in the tissues of the animals given desensitization treatment. Above all, the specificity of the effect of desensitization makes it quite certain that antibody is responsible for the *in vitro* tuberculin sensitivity of tissue explants of tuberculin-sensitive animals.

Summary. Experiments are presented which show that the *in vitro* effects of tuberculin in suppressing the initial migration of leucocytes from splenic explants of tuberculous guinea pigs is markedly reduced by specific desensitization.

This finding indicates that the cytotoxic effect of tuberculin on tissue explants of tuberculin-sensitive animals is the result of the interaction of a sessile antibody with specific antigen.

---

[10] Shwartzman, G., *Arch. Path.*, 1928, **6**, 773, 790.

16501

Purification of Mouse Poliomyelitis Virus by Methanol Precipitation at Low Temperatures.*

HELENE P. BRUMFIELD, CYRIL S. STULBERG, AND H. O. HALVORSON.
(Introduced by M. B. Visscher.)

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis.

Purification of viruses, which is essentially the separation of viral protein from non-viral protein, has been the object of a large number of investigations. In an extensive review Beard[1] has critically examined the present status of the purification of animal viruses which principally involve ultracentrifugal procedures. Cox, Van der Scheer, Aiston, and Bohnel[2] were the first to report the purification and concentration of influenza and other viruses by using organic solvents to precipitate the proteins from solutions. Recently Gollan[3] has adapted the procedures of Cox *et al.*[2] to the purification of a mouse strain of MM poliomyelitis virus from mouse brain.

This investigation concerns the purification of Theiler's GDVII strain of mouse encephalomyelitis virus by methanol precipitation, and the comparison of methanol precipitates from infected mouse brain with those obtained from

---

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Beard, J. W., *J. Immunol.*, 1948, **58**, 49.

[2] Cox, H. R., Van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

normal uninfected mouse brain.

Materials and methods. The procedures used in this investigation were similar to those developed by Cox *et al.*[2] in the purification of influenza viruses and modified by Gollan[3] in the purification of MM poliomyelitis virus. Briefly, the procedures included freezing and thawing, removal of some of the tissue lipid with ether, and the precipitation of virus protein with methanol at low temperatures. In this investigation Theiler's GDVII strain of spontaneous mouse encephalomyelitis virus was used.

Mice were infected with Theiler's virus by intracerebral inoculation. At the height of paralysis the mice were killed and their brains were recovered aseptically. The brains were weighed, ground in a mortar, and the resultant emulsion was diluted to a 20% suspension in distilled water. The tissue suspension was then subjected to additional grinding in a Potter Elvehjem homogenizer, after which it was frozen over night at –20°C. After thawing at room temperature, the suspension was centrifuged for 1 hour at 5,000 R.P.M. at 4°C. The supernatant fluid was withdrawn, frozen, thawed, and centrifuged at the same rate and temperature. The supernate was again withdrawn, dispensed in 20 ml aliquots, and stored at –20°C until used. The material obtained at this point will be referred to as crude virus suspension.

To purify the crude virus, some lipoidal material was first removed by ether extraction. Twelve ml of ethyl ether was added to each 20 ml of crude virus, and the mixture was shaken and then centrifuged at 5,000 R.P.M. for 30 minutes at 4°C. The lipid layer was removed and the aqueous virus suspension was subjected to a vacuum to remove the dissolved ether. The suspension was then frozen at –20°C, thawed, and again centrifuged for 30 minutes at 4°C. The supernatant fluid was diluted to 200 ml with 0.1 M phosphate buffer, pH 7, and cooled to –1°C. Absolute methyl alcohol, cooled to –40°C, was added drop by drop by means of a capillary pipette and with constant stirring while the suspension was kept at –1°C (to avoid heat of dilution) until the suspension contained the desired concentration of alcohol. The resultant turbid suspension was held at –4°C for 1 to 4 hours and then centrifuged for 1 hour at 5,000 R.P.M. and at 4°C. The precipitates were washed with phosphate buffers of low pH values in attempts to remove more of the non-viral protein and subsequently were placed in 0.1 M phosphate buffer at pH 7. This was frozen and thawed before the precipitate went into solution. The final suspensions appeared water-clear.

The infectivity of the crude and purified virus suspensions was tested by preparing serial 10-fold dilutions and by inoculating 0.03 ml of each dilution into each of 3 Swiss albino mice intracranially. The infective titers were determined by the 50% end-point method of Reed and Muench.[4] Total nitrogen determinations were made by the micro-kjeldahl method of both crude and purified virus suspensions. In order to obtain comparative infectivity and nitrogen values, all calculations were made on the basis of equal volumes of the crude and purified fractions.

Normal uninfected mouse brains were subjected to the same purification procedure, in which total nitrogen determinations and activity tests were also made.

The factors that might possibly be influential in affecting the activity and the relative purity of the final purified product were thought to be concentration of methanol, the pH of the suspension from which the methanol precipitation was made, and the pH and molarity of the buffers used to wash the methanol precipitates. Variations that undoubtedly would occur as a result of temperature variations were not investigated. Methanol was used in concentrations of 25% and 30%. Suspensions were precipitated at pH 7 and pH 6. The precipitated proteins were washed with phosphate buffers of pH 4.6, 5.1, and 5.6 with molar concentrations of .01 M and .02 M.

30% methanol, precipitation at pH 7. Purification, in terms of the percentage of total

---

[3] Gollan, F., PROC. SOC. EXP. BIOL. AND MED., 1948, in press.

[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.

Purification of Theiler's Virus by Methanol Precipitation.

Exp. No.	Methanol conc.	pH		Mg N per ml		% removal N	Titer (LD_{50})		MLD per mg N
		ppt'n	wash	crude	purified		crude	purified	
A1	30%	7	—*	1.52	.0386	97.5	$10^{-8.5}$	$10^{-7.5}$	1.6×10^8
A2	"	"	4.6	1.52	.33	78.0	$10^{-8.5}$	$10^{-8.5}$	1.9×10^8
F15	"	"	5.1	2.42	.026	98.9	$10^{-8.5}$	10^{-6}	7.7×10^6
F16	"	"	"	2.42	.027	98.9	$10^{-8.5}$	10^{-7}	7.4×10^7
D8	"	"	5.6	0.49	.0046	99.0	$10^{-6.7}$	$10^{-7.2}$	6.8×10^8
B4	25%	"	5.1	1.60	.195	87.8	$10^{-7.2}$	10^{-7}	1.0×10^7
C5	"	"	"	2.51	.018	99.5	$10^{-5.7}$	10^{-6}	1.7×10^7
D9	"	"	"	0.49	.0098	97.1	$10^{-6.7}$	$10^{-6.2}$	3.2×10^7
H21	"	"	"	1.38	.0256	98.2	$10^{-6.5}$	$10^{-6.2}$	1.2×10^7
C6	"	"	5.6	2.51	.112	95.3	$10^{-5.7}$	$10^{-6.2}$	2.8×10^6
J1	30%	6	5.1	1.26	.123	91.2	10^{-6}	10^{-6}	
Normal brain	"	7	"	1.56	.018	98.6			
"	"	6	"	1.56	.085	94.6			

* Precipitate was not washed.

nitrogen removed, varied from 97.5% to 99%. This variation occurred irrespective of whether the pH of the buffers used to wash the precipitates was 5.1 or 5.6. The activities of the purified samples, based on infectivities of equivalent volumes of crude and purified virus suspensions, varied from 0 to 100-fold. When crude suspension was only diluted to 40 ml and when this methanol precipitate was washed with phosphate buffer at pH 4.6, only 78.2% of the total nitrogen was removed but all of the virus was recovered. In 2 parallel purifications it was observed that remarkably close checks were obtained in the nitrogen determinations. The results are shown in Table I.

25% methanol, precipitation at pH 7. Again the percentage of nitrogen removed was subject to variation, ranging from 87.8% to 99.5% when buffers at pH 5.1 and 5.6 were used to wash the precipitate. The activities in this case, however, consistently remained undiminished, and it appeared that approximately 100% of the virus was always recovered.

30% methanol, precipitation at pH 6. On the basis of one determination slightly less nitrogen appeared to have been removed, although the methanol precipitate appeared to be considerably heavier. There was approximately 100% recovery of the virus activity, as shown in Table I.

Normal brain precipitated with 30% methanol at pH 6 and pH 7. It was interesting to observe that when normal uninfected mouse brain was subjected to the same purification procedures, the amount of nitrogen recovered in the purified product was not significantly different from some of the nitrogen values obtained in purified infected brain. Precipitation at pH 7 yielded slightly purer products. The results are presented in Table I. As would be expected, normal brain did not exhibit virus activity.

Yields. The activities of each determination appeared to vary widely in some cases. The inaccuracy of the animal titrations may be responsible in part for this variation, although it was noticed that when the methanol precipitates were held at −4°C for 4 hours, increased yields were obtained.

Conclusions. Although at first glance the data may appear rather inconsistent, closer inspection reveals that the results are consistent when consideration is given to the experimental error involved in some of the methods. The variation in the nitrogen determinations of the crude virus suspensions were thought to be due in turn to expected variation in the amount of protein removed in the freezing and thawing steps. Estimations of the degree of purity have been presented in terms of the amount of nitrogen eliminated from the crude sample. Since purified normal brain gave nitrogen values which bore a close similarity to those obtained with purified infected tissue, it can be seen that the so-called purified fractions still contain a large amount of normal brain protein. There is no doubt that the non-viral protein greatly overbalances the viral proteins in the purified fraction. However, a relatively large amount of protein is removed during the procedure, as compared with the amount removed in the ultracentrifugation methods of purification cited by Beard.[1] The activities of crude and purified virus fractions, with a few exceptions, remain fairly constant, indicating that most of the active virus is recovered. Although the titrations may not be accurate enough to determine the exact yield, they indicate that in many of the determinations 100% of the virus appeared to be recovered. The number of infectious units of virus per milligram of nitrogen depends, in part, on the original titer of the virus, and therefore is a relative value. When the methanol precipitate was held at –4°C for 4 hours the yield of virus was greater. Apparently no significant difference resulted from the use of 25% or 30% methanol. Also, the use of buffers at pH 5.1 or pH 5.6 to wash the precipitates did not seem to appreciably alter the results. One step that did result in the reduction of nitrogen was the immediate freezing of the final precipitate at pH 7 before it went into solution. Upon thawing, insoluble protein was removed by centrifugation.

16502 P

Secretion of Fructose and Citric Acid in Transplants of Rat Seminal Vesicle and Coagulating Gland.*

T. MANN,† C. LUTWAK-MANN, AND D. PRICE.

From the Department of Zoology, The University of Chicago, Molteno Institute, and Biochemical Laboratory, University of Cambridge, England.

Earlier investigations[1,2] established fructose and citric acid as secretory products of certain accessory glands of reproduction in several mammalian species; in rats the first is found in the coagulating gland and dorsal prostate, and the latter in seminal vesicle proper and ventral prostate. Further it was demonstrated that the secretion of fructose and citric acid depends upon and is regulated by, the male sex hormone; following castration there was gradual disappearance of both substances though at varying rates, and after treatment with testosterone their level was restored.[3] In the present study more evidence has been accumulated which emphasizes the effect of testosterone upon the accessory reproductive organs as regards their ability to produce fructose and citric acid. Using subcutaneous

---

* This research was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Fund of The University of Chicago.

† On Traveling Fellowship from Agricultural Research Council of Great Britain.

[1] Mann, T., *Biochem. J.*, 1946, **40**, 481.

[2] Humphrey, G. F., and Mann, T., *Nature*, London, 1948, **161**, 352.

[3] Mann, T., and Parsons, U., *Nature*, London, 1947, **160**, 294.

TABLE I.
Fructose and Citric Acid in Transplants of Combined Tissues of Seminal Vesicle and Coagulating Gland.

Male rats, I and II, implanted at the age of 50 days with 15-day-old donor tissue, autopsied 96 days later.

Rat	Wt of tissue (seminal vesicle plus coagulating gland) mg		Fructose, mg%		Citric acid, mg%	
	I	II	I	II	I	II
Organs of host	1210	1329	68	49	58	36
Transplants	1450	1400	43	24	17	60

TABLE II.
Fructose in Transplants of Coagulating Gland.

	Male host, days of age at		Fructose	
Donor, days of age	implantation	autopsy	Coagulating gland of host, mg%	Transplant of coag. gland, mg%
40	40	67	140	147
50	70	107	138	168
47	150	177	200	44

transplants of seminal vesicle and coagulating gland tissue in rats, it was shown that such transplants are capable of secreting fructose and citric acid in response to testosterone propionate, in complete anatomical separation from the rest of the reproductive tract.

The following groups of experimental animals were employed: A. 20 normal control male animals in which the content of fructose and citric acid in the seminal vesicle and coagulating gland was determined in relation to age; B. 30 male and female rats were used for transplantation experiments as hosts—normal, gonadectomized, or gonadectomized and treated with testosterone propionate (200 μg daily). The subcutaneous transplantation technic employed was that previously developed for the cytological study of rat accessory organs.[4,5] Portions of coagulating gland alone or together with seminal vesicle tissue (1-3 mg) from donors aged 15 to 50 days, were inserted into subcutaneous sites on the abdominal wall of the hosts aged 30 to 115 days. They were allowed to grow in the hosts for 16 to 96 days; then they were dissected, weighed and analyzed; for comparison the reproductive organs of the host were also assayed for fructose and citric acid.

Results. A. The accessory organs of reproduction in very young rats such as routinely used as donors, contained low quantities of fructose and citric acid; from about 40 days onward, *i.e.*, shortly before maturation of sperm, the content increased and reached a high level at 3 months of age when spermatozoa were fully active. For example, coagulating gland at 40 days had 40 mg % or 5 μg/organ, at 85 days 166 mg % or 250 μg/organ, of fructose; seminal vesicles at 40 days contained 10 mg % or 10 μg/organ, at 85 days 44 mg % or 300 μg/organ, of citric acid. These data are based in each case upon an analysis of a sample representing an aggregate of gland tissue. Glands from 8 rats were used at 40 days; from 5 rats at 85 days.

B. In transplants of combined coagulating gland plus seminal vesicle tissue, both fructose and citric acid were present (Table I), in those from coagulating gland alone only fructose was found but citric acid was absent (Table II). Coagulating gland transplants in castrated males treated with testosterone propionate for several weeks showed 144 mg %, the host coagulating glands 244 mg %, fructose; however, cessation of hormone treatment for 3 weeks caused a decline in grafts to 12 mg %, in host tissue to 5 mg %. In

---

[4] Price, D., *Physiol. Zool.*, 1941, **14**, 145.

[5] Price, D., *Anat. Rec.*, 1942, **82**, 93.

spayed or non-spayed females injected with testosterone propionate, coagulating gland transplants contained 102 to 191 mg % fructose; here too, the level in the transplants fell to 3 to 12 mg % fructose when testosterone was withheld for 3 weeks.

Summary. Subcutaneous transplants of coagulating gland and seminal vesicle in rats, were capable of producing fructose and citric acid when grown a) in normal males, b) castrated testosterone-treated males, and c) testosterone-treated non-spayed or spayed females.

Two of us (T. M. and C. L.-M.) wish to acknowledge gratefully the kind interest and hospitality offered them during their stay at the Department of Zoology, The University of Chicago, by Dr. Carl R. Moore, and the kind assistance from the Department of Biochemistry, The University of Chicago.

16503

Therapeutic Activity of Bacitracin in Rabbit Syphilis, and Its Synergistic Action with Penicillin.*

HARRY EAGLE AND RALPH FLEISCHMAN. (Introduced by J. A. Shannon.)

From the Laboratory of Experimental Therapeutics, U. S. Public Health Service, and the Johns Hopkins School of Hygiene and Public Health, Baltimore, Md.

Bacitracin is an antibiotic derived from culture filtrates of certain strains of *Bacillus subtilis*, and first described by Johnson, Anker, and Meleney.[1] As shown in a previous concentration from this laboratory,[2] it has a low renal clearance which approximates the glomerular filtration rate, in contrast to penicillin which has a renal clearance approximating the total renal plasma flow.[3] In consequence, bacitracin is excreted more slowly than penicillin, the rate of excretion remaining at a reasonably constant level for a period of several hours; and the blood level also falls more slowly than does that of penicillin G injected in similar gravimetric dosage.

In vitro, bacitracin has been shown to be actively treponemicidal against the cultured Reiter strain of *Treponema pallidum*.[4] Concentrations of 0.004 units per cc inhibited the growth of the organisms, and 0.1 unit per cc killed 99.9% in 48 hours. Against the pathogenic *T. pallidum in vivo*, doses of 72 units/kg given intramuscularly caused the temporary disappearance of the organisms from rabbit testicular chancres.[4] It may be noted that in both rabbits and man, this dosage provides plasma concentrations in excess of 0.1 unit per cc for approximately 2 to 3 hours, and in excess of 0.01 unit per cc for approximately 6 to 8 hours.[2] This period of exposure was apparently enough to render the chancre temporarily dark-field negative, but was not enough to kill all the organisms. The organisms regularly reappeared in the lesions days, weeks or even months after the injection; and it required single doses of 5,000 units/kg and more to effect the permanent healing of the primary lesion.

The present paper is a report on the therapeutic activity of bacitracin in rabbit syphilis when aqueous solutions were injected intramuscularly once daily for 4 days. Further, data are given which suggest a remarkable synergistic action between penicillin and

---

* This work was carried out in part under a research grant from National Institute of Health, U. S. Public Health Service.

[1] Johnson, B., Anker, H., and Meleney, F., *Science*, 1945, **102**, 376.

[2] Eagle, H., Newman, E. V., Greif, R., Burkholder, L. M., and Goodman, S. C., *J. Clin. Invest.*, 1947, **26**, 919.

[3] Eagle, H., and Newman, E., *J. Clin. Invest.*, 1947, **26**, 903.

[4] Eagle, H., Musselman, A. D., and Fleischman, R., *J. Bact.*, 1948, **55**, 347.

bacitracin. The relatively small numbers of animals so far tested make the results of qualitative rather than quantitative significance. The synergistic action was nevertheless striking; and the incomplete data are reported at this time because they indicate the desirability of a clinical trial of bacitracin and penicillin used in combination in the treatment of human syphilis.

Methods and materials. The method of evaluating cure in the treated rabbits by lymph node transfers to normal animals 4 to 6 months after treatment has been adequately described in previous reports from this laboratory.[5,6]

The bacitracin was obtained from the Ben Venue Laboratories, Bedford, Ohio, and the various lots assayed at 18 to 41 units per mg. The penicillin used was a commercial preparation of crystalline penicillin G, generously supplied by the Commercial Solvents Company (Lot No. 46042605), assaying at 1,500 units/mg. The dosages of penicillin indicated in Fig. 1 and Table I were adjusted for 1,667 units/mg (1.11 mg administered instead of 1, etc.).

Aqueous solutions of the two drugs were injected into the muscles of the thigh, on opposite legs, within a minute of each other; and

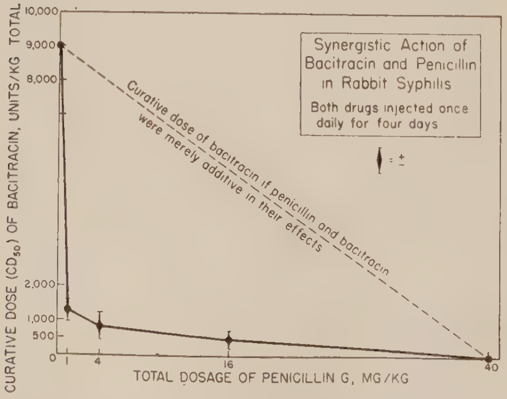


FIG. 1.

The synergism between bacitracin and penicillin with respect to the curative dose in rabbit syphilis. (From data of Table I.)

TABLE I.

Approximate Curative Dose of Bacitracin in Rabbit Syphilis, and its Synergistic Action with Penicillin. (Both drugs injected intramuscularly in aqueous solution, once daily for 4 days.)

Total penicillin dosage, mg/kg	Proportion of rabbits cured by total bacitracin dosages (units/kg) of											Approximate CD$_{50}$ dosage of bacitracin, units/kg*
	40,000	20,000	10,240	5120	2560	1280	640	320	160	80	40	
0	6/6	5/5	3/5	0/9	0/5							9200 ± 2200
1	3/3	5/6	6/6	5/5	5/6	1/3	1/4	0/5	0/2	0/4	0/2	1280 ± 310
4		4/4	4/4	4/5	6/6	1/3	0/4	2/5	1/3	0/1	0/3	840 ± 380
16		6/6	3/3	5/5	5/5	2/4	1/3	3/5	1/5	0/3	0/3	480 ± 240

* Calculated by the method of Miller and Tainter[6]: ± = standard error.†

---

[5] Eagle, H., and Hogan, R. B., *Ven. Dis. Inf.*, 1943, **24**, 69.

[6] Eagle, H., and Magnuson, H. J., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 168.

the injections were repeated once daily for 4 days.

Experimental results. Table I lists the proportion of animals cured by various dosages of bacitracin used either alone or in combination with 1, 4 or 16 mg/kg of penicillin G. The last vertical column of the table gives the CD_{50} dosages of bacitracin, calculated by the Miller-Tainter method.[7] Approximately 9,000 units/kg of bacitracin was necessary to cure 50% of the animals (CD_{50}), and the similarly curative dose of penicillin on the same schedule was on the order of approximately 30-40 mg/kg.[6] When as little as 1 mg/kg of penicillin was administered simultaneously with bacitracin, the curative dose of bacitracin was reduced from 9,200 (± 2,200)[†] to 1,280 (± 310)[†] units/kg. In other words, approximately 1/40th of the CD_{50} dose of penicillin and 1/7th of the curative dose of bacitracin were curative when used in conjunction. Further increase in the dosage of penicillin from 1 to 4 to 16 mg/kg, caused a further progressive decrease in the CD_{50} dose of bacitracin from 1,280 to 840 to 480 units/kg.

In Fig. 1, the top dotted line represents what the curative dose of bacitracin would have been were bacitracin and penicillin merely additive in their therapeutic action. The degree to which these experimental data deviate from that line is a measure of the striking synergistic action of the 2 drugs used in conjunction.

Discussion. Although both penicillin and bacitracin are curative in rabbit syphilis, the 2 drugs were as much as 7 times more effective when they were used in combination, judged by the total amounts of each necessary to effect cure. It is possible that in the treatment of human syphilis also, the synergistic use of the 2 drugs may permit a significant reduction in dosage, a significant decrease in the minimum time necessary for cure, or perhaps in both. The primary problem in the treatment of early human syphilis is, however, not the amount of treatment necessary for cure, or even the length of time for which treatment must be continued, but the fact that a significant proportion of cases are not cured by penicillin alone in whatever dosage it has yet been used.[8] Only the actual trial in man will determine whether penicillin and bacitracin are synergistic in the sense that patients not cured by penicillin or bacitracin alone may perhaps be cured if penicillin and bacitracin are used in conjunction. Pilot studies in these directions are now in progress.

Summary. Bacitracin has been shown to be effective in the treatment of rabbit syphilis. An intramuscular dose of 2,300 units/kg repeated once daily for 4 days, cured approximately half the animals tested. A striking synergism was noted in the therapeutic effect of penicillin and bacitracin. As little as 1 mg/kg of penicillin (approximately 1/40th of the CD_{50} dosage of penicillin alone) and 1,280 units/kg of bacitracin (approximately 1/7th of the CD_{50} value) were curative if used in conjunction. These data are reported because of their possible significance in relation to the treatment of the human disease.

---

[7] Miller, L. C., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, 57, 261.

[†] The standard deviation of these CD_{50} levels is large primarily because of the small number of animals so far used: the trend is nevertheless clear. The assistance of Mr. Nathan Mantel of the Statistical Section of the National Cancer Institute in calculating the CD_{50} values and their standard error is gratefully acknowledged.

[8] Merrill, M., Syphilis Symposium, Syphilis Study Section, Washington, D.C., April 8, 1948

(Continued from page iii)

MILSTONE, J. H. Activation of Prothrombin by Platelets Plus Globulin 225
MIRICK, G. S., 317.
MIXER, H., 377.
MORRISON, J. L., SAPERSTEIN, P., WALKER, H. A., and RICHARDSON, A. P. Distribution and Rate of Disposition of Myanesin in Dogs 339
NEAL, W. B., JR., 363.
NELSON, M. M., and EVANS, H. M. Effect of Desoxypyridoxine on Reproduction in the Rat 274
NECHELES, H., 248.
NOOK, M. A., 395.
OLMSTED, J. M. D., 257.
PAFF, G. H. Influence of pH on Growth on Bone in Tissue Culture 288
PARMER, L. G. On the Relative Efficacy of Emetine in Intestinal and Hepatic Amebiasis 362
PERERA, G. A., 286.
PETERSON, O., 240.
PINES, K. L., PERERA, G. A., VISLOCKY, K., and BARROWS, A. D. Effect of Adrenal Cortical Extract in Hypertensive Subjects 286
PINKERTON, H., 228.
PONKA, J. L., and LAM, C. R. Effect of Asphyxia on Blood Coagulation in Dogs 334
PORTER, W. B., 240.
PRICE, D., 413.
PUCHER, G. W., 294.
RATNOFF, O. D., and MIRICK, G. S. Influence of Diet, Sex, and Testosterone Propionate on Toxicity of Monocrotaline in Rats 317
REAGAN, R. L., LILLIE, M. G., HAUSER, J. E., and BRUECKNER, A. L. Response of the Syrian Hamster to the Virus of Newcastle Disease 293
REESE, J. D., and FOWLER, E. M. Morphology of *Hemobartonella muris* as Revealed by Darkfield Microscopy 276
REID, M. E. Gastrointestinal Tract of Guinea Pig and Elimination of Ascorbic Acid Given Intraperitoneally 403
RICHARDSON, A. P., 339.
ROGERS, G. R., 363.
ROJAS, C., 300.
SADHU, D. P., 255.
SALIS, H. Erythrophagocytosis in Bone Marrow Culture with Relation to Antagonism of Pteroylglutamic Acid 382
SAPERSTEIN, P., 339.
SCHECHTMAN, A. M. Organ Antigen in the Early Chick Embryo 263
SCHUBERT, J., 233.
SCHULTZ, E. W., and WHITE, S. C. Infectivity of Murine SK Strain of Poliomyelitis Virus 266
SHARP, D. G., 309.
SLATER, E. A., 282.
STANSLY, P. G. Studies on Polymyxin: An Assay Method for Blood and Urine 301
STULBERG, C. S., SLATER, E. A., BRUMFIELD, H. P., and HALVORSON, H. O. Purified Human Poliomyelitis Virus: Infectivity for Cynomolgus Monkeys 282
STULBERG, C. S., 410.
TAYLOR, M. J., 336.
TOBIAN, L., JR., 285.
TRAVIS, B. L., 246.

TRUSCOTT, B. L., and SADHU, D. P. Vitamin A and Thyroid Iodine After Reticuloendothelial Cell Block 255

URIST, M. R., BUDY, A. M., and McLEAN, F. C. Species Differences in the Reaction of the Mammalian Skeleton to Estrogens 324

UYEYAMA, K., 296.

VANDERBROOK, M. J., 395.

VENNESLAND, K., EBERT, R. H., and BLOCH, R. G. *In Vitro* Effect of Streptomycin and Paraaminosalicylic Acid (PAS) on the Growth of Tubercle Bacilli 250

VICKERY, H. B., PUCHER, G. W., and LEAVENWORTH, C. S. Artificial Enrichment of Beet Root Tissue with Glutamine 294

VISLOCKY, K., 286.

WALKER, H. A., 339.

WANGENSTEEN, O. H., 369.

WEDD, A. M., and BLAIR, H. A. Effect of Digitoxin on Contractility of Ventricular Muscle Strips 304

WEIL, M. L., BEARD, D., and BEARD, J. W. pH Stability, Response to Antibiotics and Factors Influencing Egg-Culture of Mumps Virus 308

WEIL, M. L., BEARD, D., SHARP, D. G., and BEARD, J. W. Purification and Sedimentation and Electron Micrographic Characters of the Mumps Virus 309

WEISER, R. S., 407.

WEISS, P., 380.

WELCH, E., 332.

WHITE, S. C., 266.

WILEY, J. L., 395.

WOODS, R. P., 380.